Investigation of Cytochrome P450 Enzymes as Biocatalysts for Multifunctional C-H Oxidation; and a Case Study of a Graduate/Undergraduate Laboratory Exchange Program

by

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DEDICATION

In loving memory of my grandmother, Dorothy D’Angelo.

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LIST OF ABBREVIATIONS

10-dml..... 10-deoxymethynolide

Å.....Angstrom

AIBN ..... Azobisisobutyronitrile

Aq .....Aqueous

AsnRS .....Asparaginyl-tRNA Synthetase

Asp ..... Aspartic Acid

Bn .....Benzyl

Bu ..... Butyl

C#.....Carbon Number

CoA.....Coenzyme A

DBU ..... 1,8-diazabicyclo(5.4.0)undec-7-ene

DCC .....*N*,*N'*-dicyclohexylcarbodiimide

DCM..... Dichloromethane

DH.....Dehydratase Domain

DIBALH..... Diisobutylaluminium Hydride

DMAP.....4-dimethylaminopyridine

DMB..... 2,4-dimethoxybenzyl

DMP .....Dess-Martin Periodinane

Et..... Ethyl

Et_3N.....Triethylamine

EtOAc ..... Ethyl Acetate

FAD.....Flavin Adenine Dinucleotide

G6P.....Glucose 6-Phosphate

G6PDH..... Glucose-6-Phosphate Dehydrogenase

Glu ..... Glutamic Acid

Hex..... Hexanes

His.....Histidine

HIV-1.....Human Immunodeficiency Virus Type 1

HPLC ..... High-Performance Liquid Chromatography

HWE.....Horner-Wadsworth-Emmons Olefination

Ipc..... Isopinocampheyl

k_{cat}..... Turnover Number

K_m ..... Michaelis Constant

KR.....Ketoreductase Domain

LCMS..... Liquid Chromatography-Mass Spectrometry

LDA..... Lithium Diisopropylamide

Leu.....Leucine

m..... Meta

M ..... Molar

***m*CPBA**.....*meta*-Chloroperoxybenzoic Acid

MD.....Molecular Dynamics

Me ..... Methyl

MIC ..... Minimum Inhibitory Concentration

Min ..... Minutes

MK ..... Menaquinone

Mol ..... Moles

MS ..... Molecular Sieves

NADH ..... Nicotinamide Adenine Dinucleotide (reduced form)

NADPH ..... Nicotinamide Adenine Dinucleotide Phosphate (reduced form)

NMR ..... Nuclear Magnetic Resonance Spectroscopy

NOESY ..... Nuclear Overhauser Effect Spectroscopy

NOS ..... Nature of Science

NRC ..... National Research Council

NRPS ..... Non-Ribosomal Peptide Synthetase

NSF ..... National Science Foundation

o ..... Ortho

***p*-PTS** ..... Pyridinium *para*-Toluenesulfonate

p ..... Para

PCC ..... Pyridinium Chlorochromate

PDC ..... Pyridinium Dichromate

PhH ..... Benzene

PhMe ..... Toluene

Piv ..... Pivaloyl

PKS ..... Polyketide Synthase

PMB ..... *para*-Methoxybenzyl

ppm ..... Parts Per Million

Pro ..... Proline

PUI ..... Primarily Undergraduate Institution

RCM ..... Ring Closing Metathesis

REACH ..... Research Experiences Across Cultures at Hope

REU ..... Research Experiences for Undergraduates

RISE ..... Research Internships in Science and Engineering

RNAP ..... Ribonucleic Acid Polymerase

RT ..... Room Temperature

S ..... Streptomyces

Ser ..... Serine

sp ..... Species

STEM ..... Science, Technology, Engineering, and Mathematics

Stl ..... Streptolydigin

***t*-BuOK** ..... Potassium *tert*-Butoxide

TASSEP ..... TransAtlantic Science Student Exchange Program

TBAF ..... Tetrabutylammonium Fluoride

TBDPS ..... *tert*-Butyldiphenylsilyl

TBHP ..... *tert*-Butyl Hydroperoxide

TBS ..... *tert*-Butyldimethylsilyl

TES ..... Triethylsilyl

TFA ..... Trifluoroacetic Acid

TFBQ Tetrafluoro-1,4-benzoquinone

THF ..... Tetrahydrofuran

Thr ..... Threonine

TIPS ..... Triisopropylsilyl

Tir ..... Tirandamycin

TLC ..... Thin Layer Chromatography

TMEDA ..... Tetramethylethylenediamine

TMS ..... Trimethylsilyl

Ts ..... Tosyl

TTN ..... Total Turnover Number

VRE ..... Vancomycin-Resistant *Enterococcus faecalis*

ZPD ..... Zone of Proximal Development

ABSTRACT

Cytochrome P450 monooxygenases are ubiquitous in nature, catalyzing a variety of oxidative transformations. Recent work has shown that the bacterial P450 PikC can catalyze the regioselective hydroxylation of both small molecule and larger macrolactone ring systems via a unique anchoring mechanism. The catalytic versatility of PikC, however, remains limited primarily to hydroxylation reactions. Further research into the activity of mixed function P450s could therefore expand upon the use of enzymes as biocatalysts.

Chapters 1-3 of this thesis focus on the rapid synthesis of a series of analogs of tirandamycin, the natural substrate of the multi-functional P450 TamI, to access novel oxidation products using either PikC or TamI. Investigation into these biocatalytic systems explores whether PikC can install multiple types of oxidative functionality within the bicyclic core of tirandamycin as well as probes the utility of TamI as a mixed function biocatalyst for site-selective oxidation of unnatural substrates.

Chapter 4 of this thesis describes a case study of a laboratory exchange between a chemistry graduate student from a R1 research institution and an undergraduate student involved in summer research at a primarily undergraduate institution (PUI). Interviews and observations were used as a means to document learning of participants. Findings from this study show an increase in personal and

professional growth among exchange students and provide support for the design and implementation of these programs by others.

CHAPTER I

Isolation, Biological Activity & Biosynthesis of Tirandamycin

1.1 Tetramic Acid Natural Products

The antibiotic tirandamycin is one of several naturally occurring tetramic acids that are characterized by the inclusion of a 2,4-pyrrolidinedione ring system (Figure 1.1). While this functional motif has been known since its original synthesis in the early twentieth century, the pharmacological importance of the tetramic acid was not well understood until the isolation of bio-active tetramic acid-containing natural products in the 1960s.[1] Owing largely to their breadth in structural and biological diversity, tetramic acids have now become a topic of renowned interest to many synthetic and medicinal chemists.

Tirandamycin is one of several known dienoyltetramic acids which contain a 1-oxopentadienyl tether linking the tetramic acid at the C3 position to a bicyclic ketal moiety. Other structurally related members of this family vary in their substitution around both the bicyclic and tetramic acid ring systems. For example, the first reported dienoyltetramic acid streptolydigin,[2] (Figure 1.1) features a spirocyclic epoxide appended to the bridge of the bicycle and a glycosylated tetramic acid bearing an amide side chain. Tirandalydigin,[3] which can be considered a structural hybrid of tirandamycin and streptolydigin, maintains the same bicyclic moiety as streptolydigin, but lacks the

presence of further functionalization around the tetramic acid center. Nocamycins I and II,[4,5] on the other hand, possess the same unsubstituted acyl tetramic acid subunit connected to a structurally distinct fused oxolane ring system.

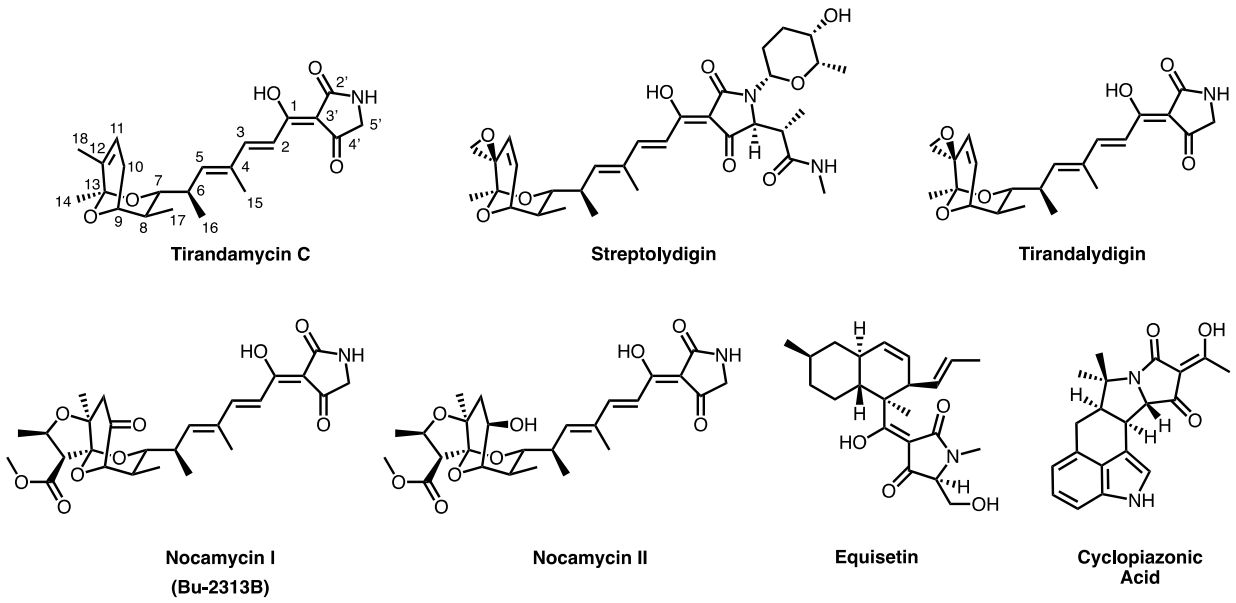


Figure 1.1 – Select Examples of Tetramic Acid Natural Products

While the dienoyltetramic acids have received notable attention due to the antibiotic activity conferred by the presence of the 3-dienoyl subunit, tetramic acid natural products display a diverse array of structural and biological properties. The fungal metabolite Equisetin, for example, was found to be a novel *in vitro* HIV-1 integrase inhibitor, leading to its evaluation as a potential chemotherapeutic.[6] Furthermore, α-cyclopiazonic acid is a tetramic acid-containing mycotoxin that displays disparate biological effects, including alteration of neurotransmitter levels,[7] inhibition of calcium uptake,[8,9] and antioxidant activity.[10]

The remainder of this chapter will summarize the isolation, biological activity and proposed biosynthetic pathways of tirandamycin, which provide a foundation for the work presented in chapters 2 and 3 of this thesis.

1.2 Bacterial Isolation of Tirandamycin

Tirandamycin was first isolated by Meyer in 1971 from the terrestrial bacterium *Streptomyces tirandis*.[11] At the time, the structure was thought to be similar to that of streptolydigin, although the absolute configuration of tirandamycin was not confirmed until later in 1973 from the x-ray crystal of the *p*-bromophenacyl ester of tirandamycic acid.[12] Three years later, tirandamycin was isolated again from *Streptomyces flaveolus*, along with a closely related natural product bearing an additional hydroxyl group.[13] Once it was confirmed that two unique variants of tirandamycin had been identified, the original and newly isolated structures were distinguished as tirandamycin A (TirA) and B (TirB), respectively (Figure 1.2).

Subsequent work by Sherman and co-workers utilized XAD-16 resin to harvest two new secondary metabolites, tirandamycin C (TirC) and D (TirD), from the tirandamycin pathway.[14] These compounds, which were obtained from early stationary phase fermentation of the marine isolate *Streptomyces* sp. 307-9, were hypothesized to be key biosynthetic intermediates which precede TirA and TirB in the biosynthesis of tirandamycin. This was most clearly evidenced by the isolation of TirA and TirB in the absence of adsorbent resin, which was capable of sequestering and protecting TirC and TirD from further enzymatic modification. Later in 2011, Sherman et al. identified another tirandamycin congener, tirandamycin E (TirE), while further elucidating the role of the enzymes TamI and TamL in the oxidative tailoring of tirandamycin.[15] When TirC

was incubated with the recombinant P450 TamI, they observed the formation of the C10 hydroxyl to give TirE instead of TirD. These experiments, in conjunction with the characterization of the biosynthetic gene cluster from *Strep.* sp. 307-9[16], provided initial insight into the putative biosynthetic pathway from this bacterial species, which involves a multi-functional iterative cascade which resolves the differences in oxidative complexity leading from TirC to TirB. The more recent isolation of tirandamycin K (TirK), the first linear tirandamycin derivative, from a *tamI* disruption mutant strain of *Strep*. sp. 307-9, has since added to this understanding of tirandamycin biosynthesis, which will be explained in more detail in section 1.4.2 below.[17]

Over the past decade work from Ju and Shen has contributed to the identification of several additional tirandamycin species. In 2010 TirA and TirB were also isolated from *Strep.* sp. SCSIO 1666, a marine-derived actinomycete bacteria obtained from samples of sediment from the South China Sea.[18] This prompted further investigation of the tirandamycin gene cluster from *Strep*. sp. SCSIO 1666,[19] which was shown to share similar organization to that of *Strep.* sp. 307-9. Gene inactivation experiments of P450 encoding *trdI* led to the accumulation of TirC, along with a trace amount of a new product labeled by Ju and co-workers as tirandamycin C2. Given that tirandamycin C2 is the C10 epimer of TirE, for the remainder of this thesis document tirandamycin C2 will be referred to as (*R*)-TirE, with TirE alluding to the *S* stereoisomer isolated by Sherman in 2011.[15] This *S* stereoisomer (labeled as tirandamycin F by Ju) was also isolated around the same time from *Strep.* sp. SCSIO 1666 following further gene disruption studies involving a ΔtrdL mutant.[20] Without the use of XAD-16 resin however, a new compound, tirandamycin F2 (TirF2), was produced instead. (Note that this is not the

name given by Ju, who refers to this congener as tirandamycin E.) Pre-tirandamycin, an early biosynthetic intermediate which is hypothesized to precede TirC, was isolated a year later through in vivo *trdE* inactivation.[21]

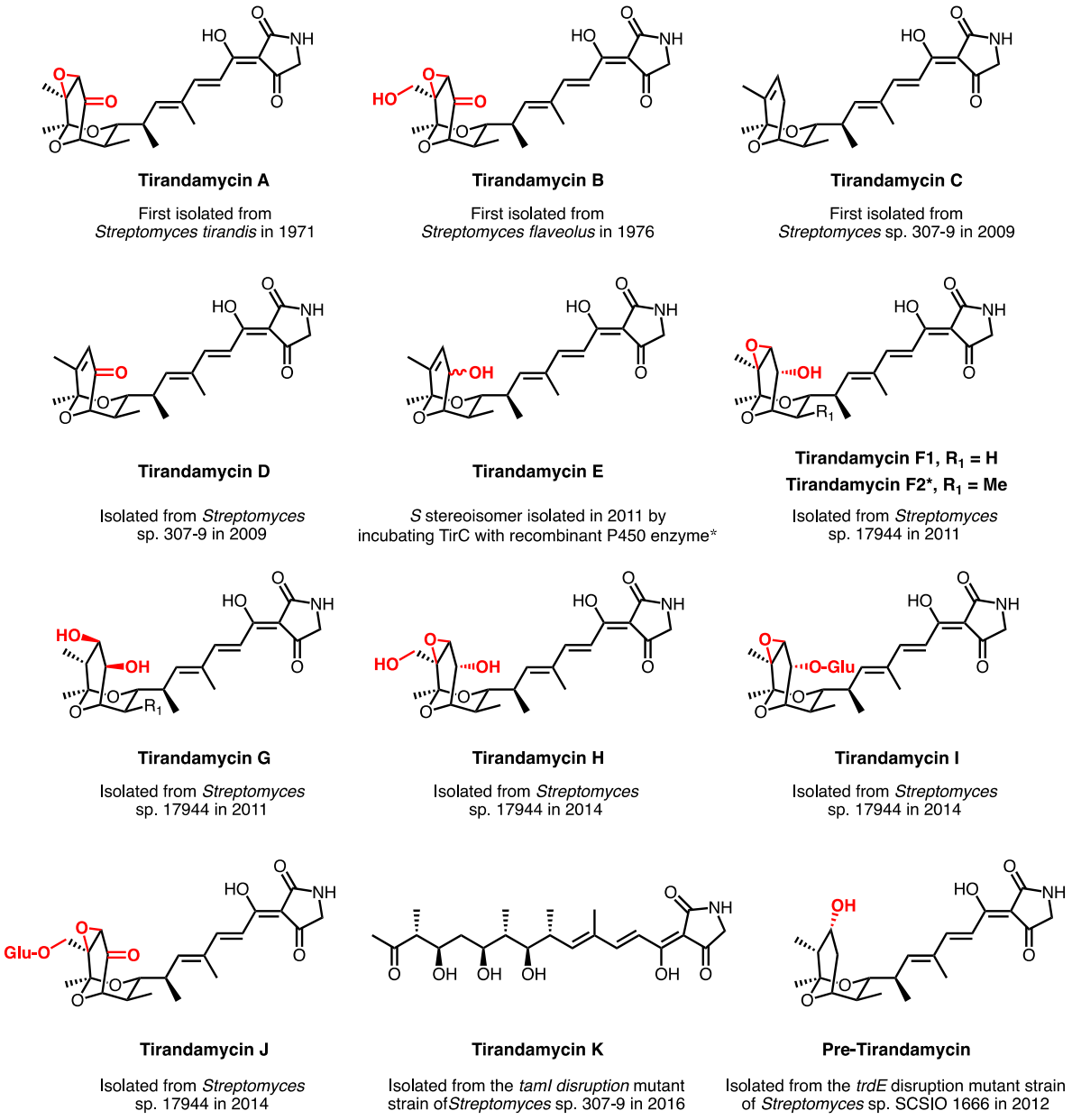


Figure 1.2 – Isolated Congeners of Tirandamycin

Tirandamycin has also been isolated from a bacterial species of terrestrial origin, *Strep.* sp. 17944.[22,23] A high-throughput drug discovery screen reported in 2011 by Shen and others led to the recovery of three unique tirandamycins, TirF1, TirF2 and TirG, from this bacterial strain in addition to TirA and TirB. (Note that TirF1 and TirF2 are referred to by Shen as TAM F and TAM E, respectively.) Interestingly, TirF1 and TirF2 share the same oxidative modification, with TirF1 lacking the C18 methyl that has been present in all other isolated triandamycin congeners to date. A year later, growth medium optimization led to the isolation of three additional biosynthetic intermediates, TirH, TirI, and TirJ from *Strep.* sp. 17944. While TirH was identified as the C18 hydroxylated analog of TirF2, spectral characterization of Tir I and TirJ showed the presence of a glucose sugar appended to the bicyclic ketal moiety, giving TirF2-10-α-D-glucoside and TirB-18-α-D-glucoside.

More recently it has been reported that TirA and TirB have also been isolated by Zhu et al. from another marine-derived bacterial species, *Strep.* sp. 17944, obtained from samples of the marine sponge *Gelliodes carnosa* from the South China Sea.[24] Notably, TirA and TirB were isolated alongside other structurally diverse natural products including a new peptide antibiotic, quinomycin G, and TirB was isolated as its tautomer in the 1-keto-4'-enol form.

1.3 Biological Activity of Tirandamycin and Related Tetramic Acid Antibiotics

1.3.1 Antibiotic Activity

Shortly following the isolation of TirA by Meyer[11], microbiologists became interested in investigating its biological activity, particularly due to its structural similarity to the known antibiotic streptolydigin. Not surprisingly, TirA was also found to inhibit the

growth of a variety of Gram-positive bacteria, although its potency was markedly less in comparison.[25,26] Later in 2009 this activity was evaluated across other biosynthetic intermediates in the tirandamycin pathway.[14] In a study conducted by Sherman and co-workers which measured activity against vancomycin-resistant *Enterococcus faecalis* (VRE), TirA was found to be the most effective antibiotic with a minimum inhibitory concentration (MIC) of 2.25 μM in comparison to TirB (MIC = 100 μM), TirC (MIC = 110 μM) and TirD (MIC = >9 μM). This showed that while oxidative modification to install the C10 ketone and C11/C12 epoxide results in an increase in potency, the introduction of the C18 hydroxyl attenuates this activity. Notably, tirandamycin A is also the most readily isolated tirandamycin congener,[15] suggesting that the tirandamycin pathway is tuned towards the production of its most bioactive intermediate. Furthermore, measurement of the antibiotic activity of linear TirK against several Gram-negative and Gram-positive strains of bacteria showed a significant decrease in potency with respect to TirC, indicating that the tirandamycin bicycle likely contributes to its antmicrobial properties.[17]

Early studies on the antibacterial activity of TirA prompted further investigation into its biological mechanism. Work by Reusser[25,27] showed that similar to streptolydigin, TirA inhibits the *de novo* synthesis of RNA in *E. coli*, interfering with the chain initiation and chain elongation processes catalyzed by RNA polymerase (RNAP). Reusser also reported that this inhibition was caused by interactions of tirandamycin with the polymerase itself, rather than the DNA template or template-enzyme complex. An analogous effect, however, was not observed on mammalian RNAP derived from rat liver.

More extensive research has explored the details of RNAP inhibition by streptolydigin, which as mentioned above, is hypothesized to be related to the mechanism of action of tirandamycin. Crystal structures of the streptolydigin-RNAP complex[28,29] have revealed that three main RNAP structural elements come in contact with streptolydigin: the streptolydigin (Stl) pocket and bridge helix, which interact with the streptolydigin bicycle and its 1-oxopentadienyl tether, and the trigger loop region, which associates with streptolydigin's highly substituted tetramic acid (Figure 1.3 and 1.4). Notably, the Stl tetramic acid moiety is also positioned near the DNA non-template strand in the transcription elongation complex (Figure 1.3), suggesting that incorporation of a DNA binding substituent could increase the potency of future analogs of streptolydigin.

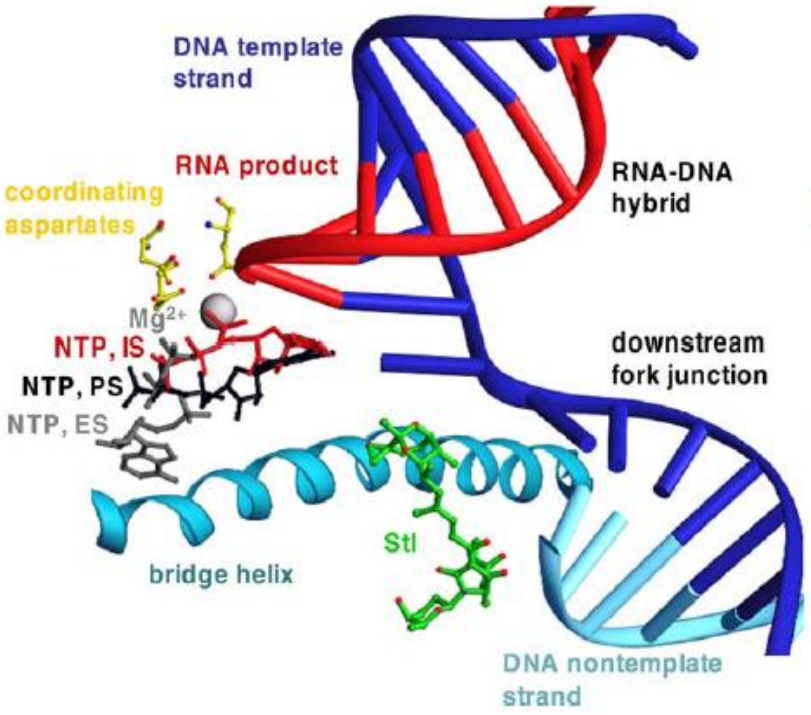


Figure 1.3 – Modeled Interactions of Streptolydigin (Stl) with RNAP Transcription Elongation Complex[28]

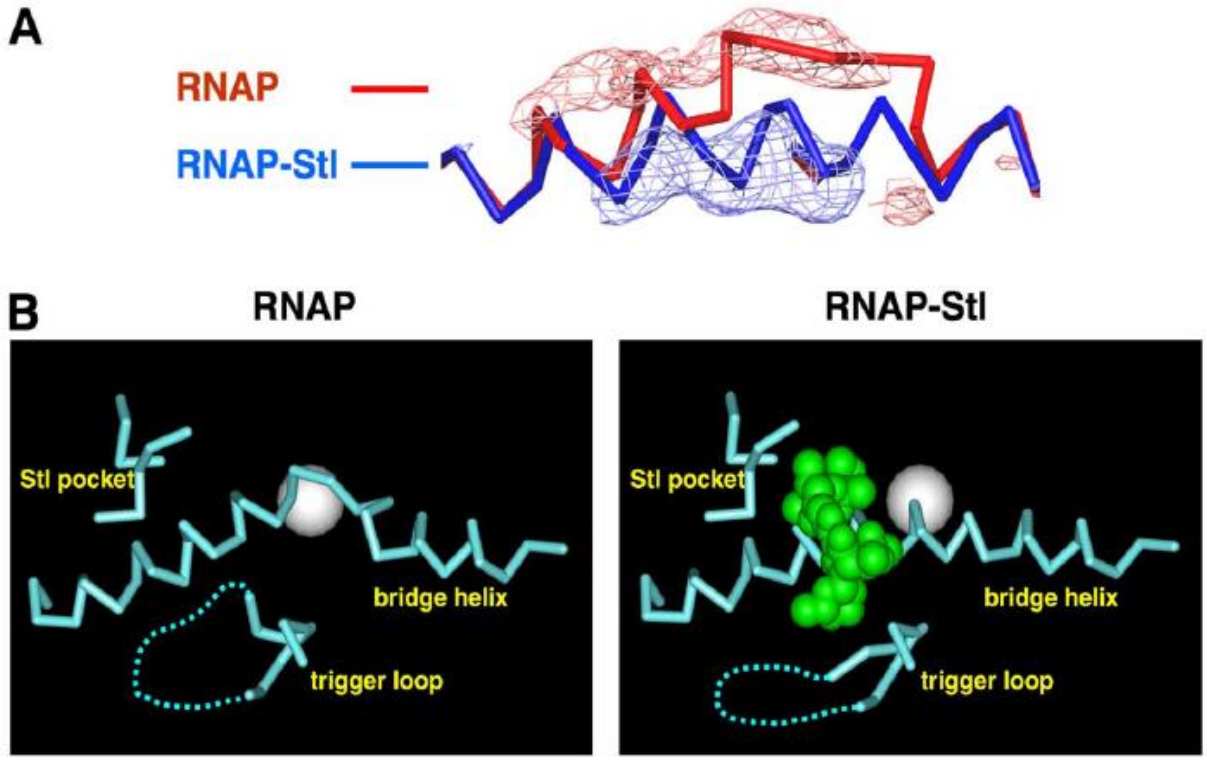
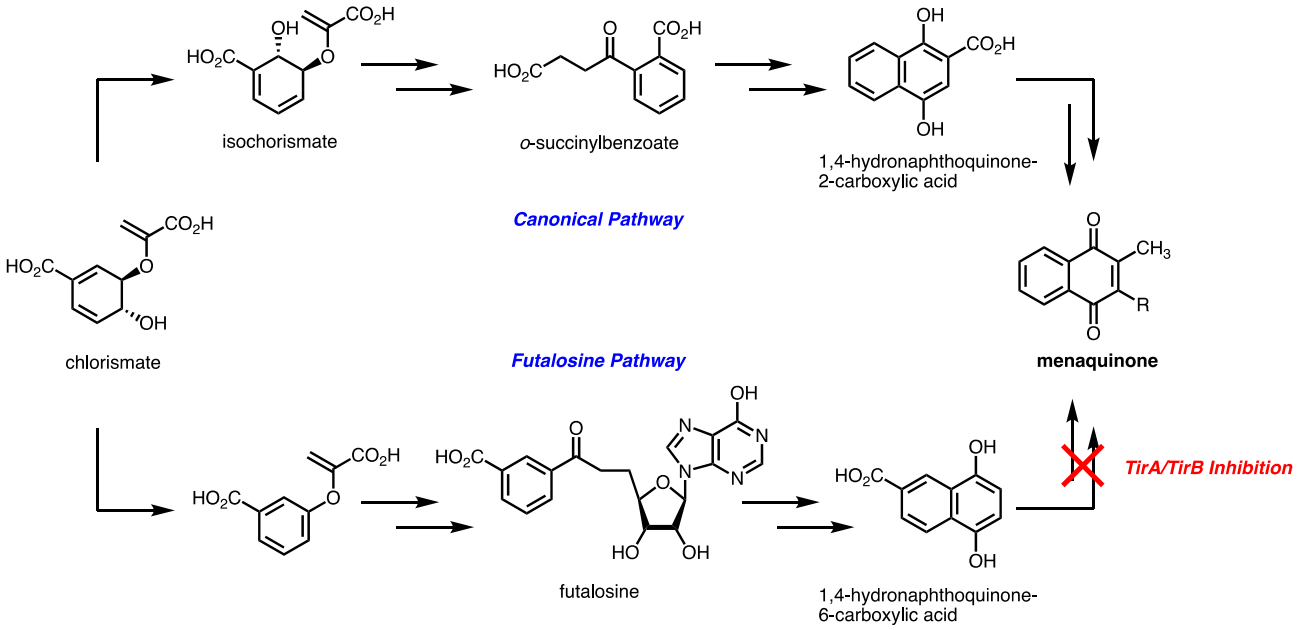


Figure 1.4 – Structural Basis of Inhibition by Streptolydigin (Stl) - Bridge helix and trigger loop conformational differences between RNAP and RNAP-streptolydigin complex[28] (*In figure B the white sphere represents the Mg^{2+} active-center and the green is Streptolydigin*.)

It has been proposed in the literature that RNAP function is dependent on the alternation of the RNAP bridge helix between its straight and bent conformational states.[30–33] Comparison of Stl-bound and unbound RNAP illustrates the impact that streptolydigin has on these active site conformations, forcing the bridge helix to adopt a straight rather than bent orientation (Figure 1.4).[28] This also causes the opening of the trigger loop, which must be displaced in order to accommodate Stl, leading to an inactive configuration of the elongation complex.[34] It is therefore likely that streptolydigin binds to and stabilizes RNAP in this orientation, inhibiting the conformational cycling thought to be necessary for RNAP function.

Recently Dairi disclosed that tirandamycin also inhibits the synthesis of menaquinone (MK), or vitamin K2, in *Bacillus halodurans* C-125.[35] MK is essential to the survival of many bacterial strains, being produced via one of two different pathways from a common biochemical intermediate (Scheme 1.1). TirA and TirB were found to exclusively target the futalosine pathway, likely disrupting MK synthesis following the formation of 1,4-hydronaphthoquinone-6-carboxylic acid. Notably, the futalosine pathway is the operative pathway in *Helicobacter pylori*, Gram-negative bacteria linked to the development of ulcers and stomach cancer. Since humans and useful intestinal bacteria lack this alternative pathway, futalosine pathway inhibitors such as tirandamycin can inspire the design of novel gastrointestinal therapeutics.



Scheme 1.1 – Inhibition of Futalosine Pathway by Tirandamycin (R = isoprenoid side chain of varying length)

1.3.2 Antiparasitic Activity

As alluded to earlier, the isolation of tirandamycin from *Strep.* sp. 17944 was the result of a natural product drug discovery program which identified *Strep*. sp. 17944 as an active strain against the filarial parasite *Brugia malayi*.[22] Specifically, TirB inhibits *B. malayi* asparaginyl-tRNA synthetase (AsnRS), which is expressed in all developmental stages of the parasite and is necessary for its survival. Infection by *B. malayi* roundworms causes disruption of the lymphatic system in humans, leading to the development of the tropical disease lymphatic filariasis.[36] Today, 856 million people in 52 countries remain at risk of suffering from lymphatic filariasis, creating a need for new antifilarial drugs to combat this global health problem.

Shen, Kron and co-workers evaluated the inhibitory activity of tirandamycin against *B. malayi* AsnRS through a "pretransfer editing assay" designed to measure AsnRS activity through pyrophosphate production.[37] While all other tirandamycin congeners (TirA, TirF1, TirF2, TirG, TirH, TirI and Tir J) showed no measurable impact on phosphate generation, TirB was found to selectively inhibit *B. malayi* AsnRS over human AsnRS with an IC_{50} of 30 μM.[22,23] This suggests the high level of oxidative tailoring present in TirB is a critical pharmacophore, as reduction of the C10 carbonyl (TirH) or glycosylation of the C18 hydroxymethyl (TirJ) completely eliminates any observed antifilarial activity.

TirB was also shown to kill *B. malayi* worms *in vitro* with a greater efficiency than albendazole, an approved drug for the treatment of lymphatic filariasis. While more than ten days were needed for 100 μM albendazole to kill adult worms, less than 24 hours was needed for TirB (IC_{50} = 1 μM) to cause filarial death at the same

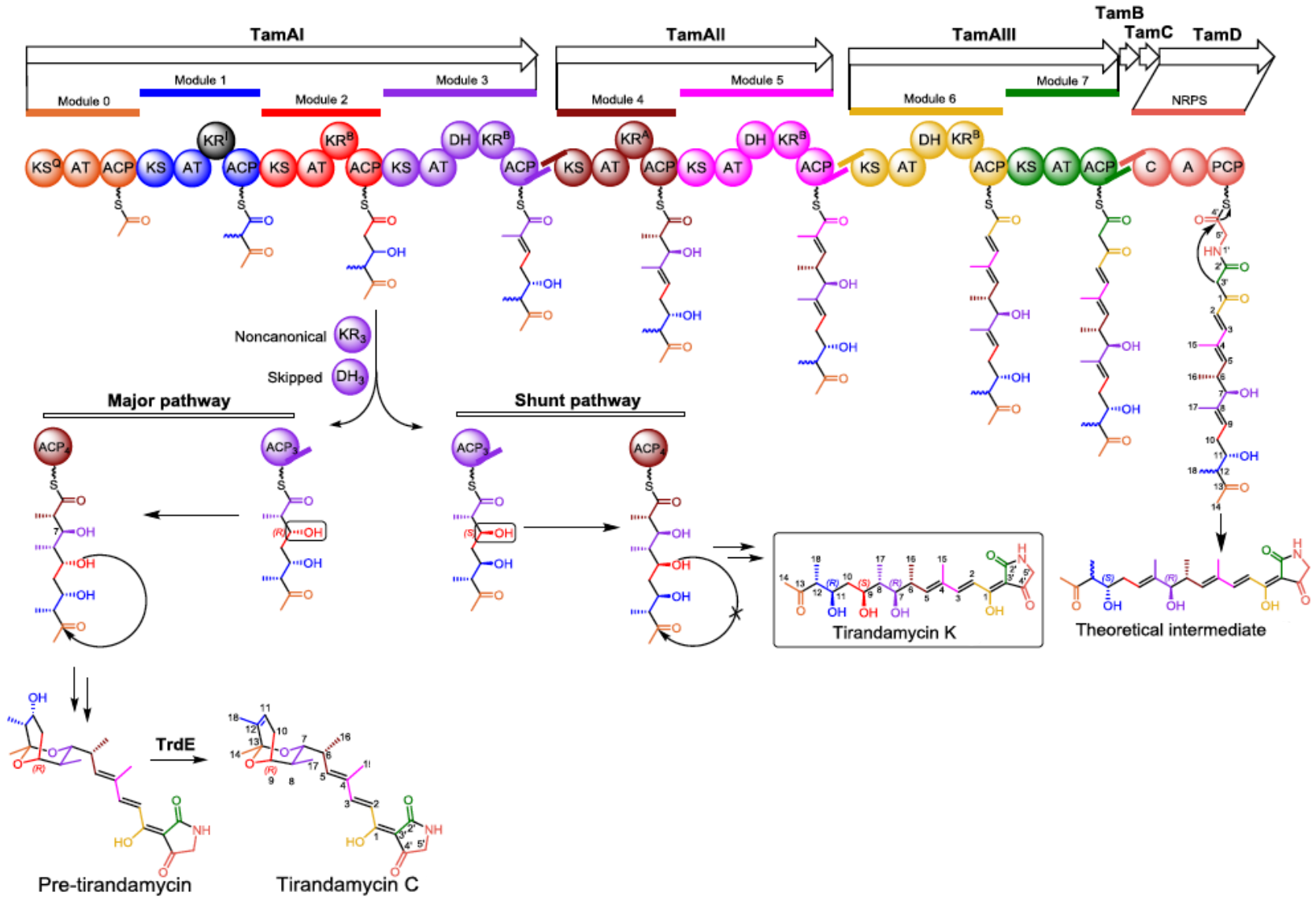
concentration. This further illustrates the potential for TirB to serve as a lead scaffold for future antifilarial drug development, which can help stop the spread of parasitic infection and contribute to the global eradication of lymphatic filariasis.

1.4 Biosynthesis of Tirandamycin

To date, the tirandamycin biosynthetic gene cluster has been independently cloned and characterized from two different bacterial species, *Strep.* sp. 307-9[16] and *Strep.* sp. SCSIO1666.[19] Both gene clusters share a similar organization, containing a hybrid polyketide synthase (PKS) / non-ribosomal peptide synthetase (NRPS) assembly-line system which is collinearly organized and is flanked by coding regions for two oxidative tailoring enzymes. This high level of similarity is also shown in the proposed biosynthetic pathways of these two bacterial species, which include protein homologs with 98% to 100% sequence identity. The remainder of this chapter will focus primarily on the *tam* gene cluster and the biosynthesis of tirandamycin from *Strep.* sp. 307-9, but will incorporate discussion of the biosynthetic pathways proposed by Ju and Shen when necessary to account for the formation of tirandamycin congeners F-J.

1.4.1 Biosynthesis of Tirandamycin C

Type 1 polyketide synthases are large, modular proteins responsible for the incorporation and modification of short chain acyl groups into a growing polyketide chain The tirandamycin biosynthetic cluster consists of three PKS genes (*tamAI*, *tamAII* and *tamAIII*), which build the skeletal framework of tirandamycin through the successive condensation of malonyl-CoA and methylmalonyl-CoA building blocks (Scheme 1.2).[16,17]



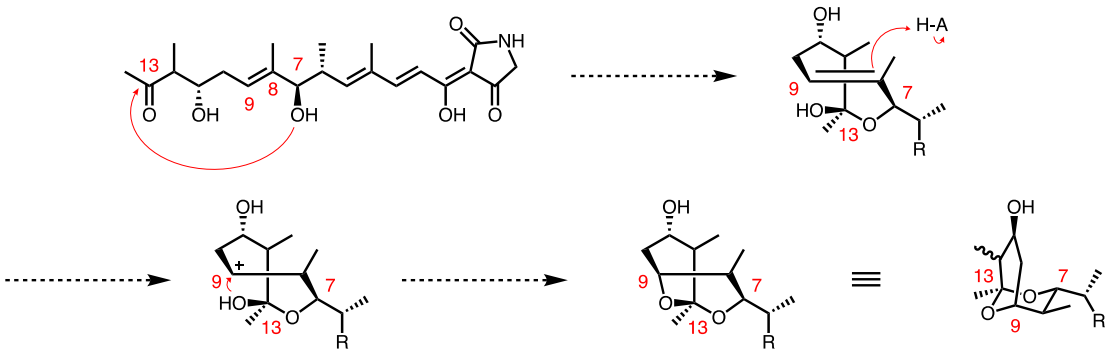
Scheme 1.2 – Hybrid PKS-NRPS Assembly Line[17]

A non-ribosomal peptide synthetase encoded by the *tamD* terminal module then allows for the introduction of glycine to the nascent polyketide chain. Unlike other assembly line enzymes, however, TamD lacks a C-terminal releasing domain, leaving the chain release mechanism of tirandamycin from the terminal module unclear. Both Sherman[16] and Ju[19] have proposed that this step involves the non-enzymatic attack of the activated C-3' methylene on the PCP thioester to give the resulting tetramic acid (Scheme 1.2), although this has not yet been confirmed experimentally.

Several different mechanisms have been suggested in the literature regarding the biosynthesis of the tirandamycin bicycle. Sherman initially hypothesized that cyclization occurs on the predicted linear intermediate that is theoretically formed following chain release from the NRPS module (Scheme 1.2).[16] Identification of the terpene synthase homolog *tamF* led to the assumption that this happens in a manner similar to terpene cyclization, involving protonation of the C8-C9 double bond followed by addition of the hemiketal hydroxyl at C9 (Scheme 1.3). A *tamF* disruption strain, however, did not lead to the accumulation of pre-cyclization intermediates as would be predicted under this mechanism, making it unlikely that bicyclic ketal formation occurs in this fashion.

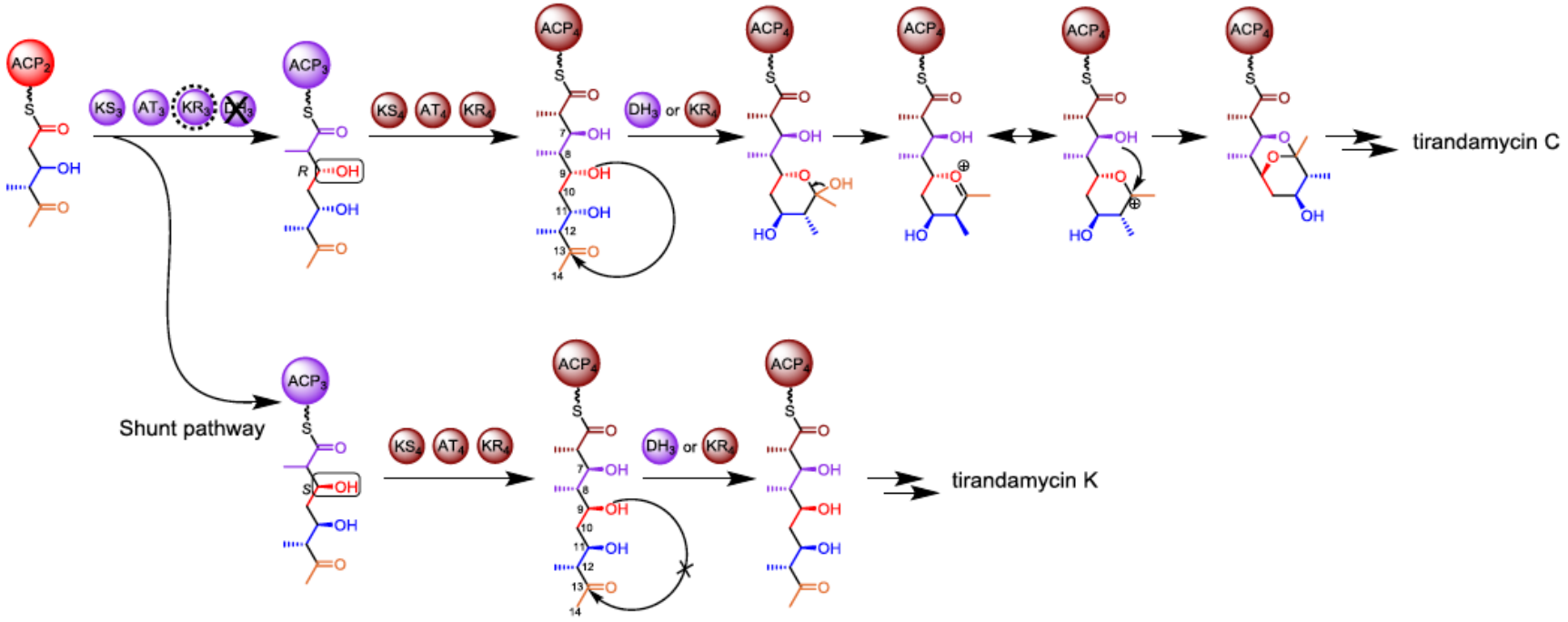
Recently, isolation of the linear tirandamycin derivative TirK prompted a second look at the proposed biosynthetic pathway of tirandamycin.[17] Notably, TirK retains the C9 hydroxyl group which was originally thought to be removed by the module 3 dehydratase domain (DH_3, Scheme 1.2). This suggests another possible role for DH_3, such as involvement in the formation of the bicyclic ketal, as initially proposed by Ju et al.[19] In this mechanism, the C9 hydroxyl, instead of the C7 hydroxyl, takes part in the

initial cyclization event, followed by dehydration and attack of the C7 alcohol on the resulting oxonium ion (Scheme 1.4). Incorporation of the C9 oxygen is in agreement with previous oxygen labeling studies on the biosynthetic pathway of streptolydigin, providing further support for this mechanism.



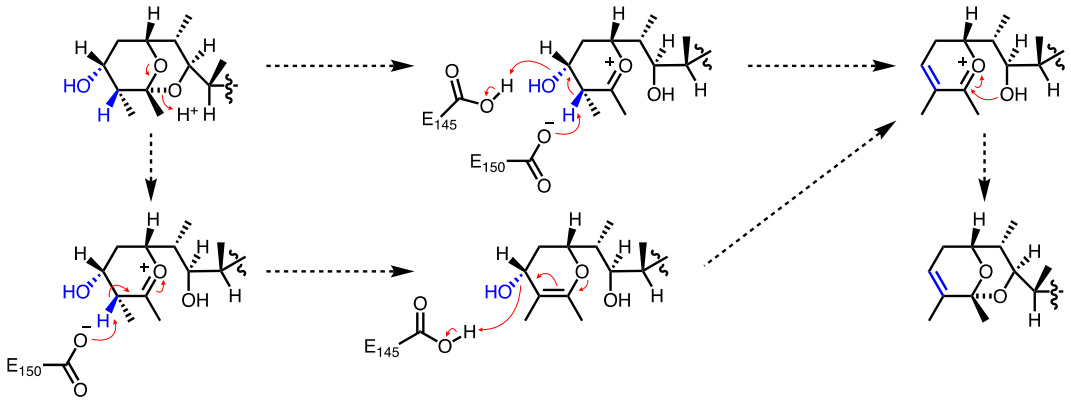
Scheme 1.3 – Proposed Mechanism of Bicycle Formation by TamF Terpene Synthase

Since the participation of DH_3 would require transfer of the pentaketide intermediate back to module 3, the module 4 ketoreductase (KR_4) also must be considered as the active site for catalysis (Scheme 1.4). Additionally, the stereochemistry of the C9 hydroxyl in TirK is opposite of what is expected by a B-type ketoreductase domain (KR_3), suggesting the reduction proceeds with non-canonical stereoselectivity to form both *R* and *S* epimers. This provides rationale as to why TirK may avoid cyclization, presuming the *9S* configuration prevents the intermediate from adopting the correct orientation for nucleophilic addition.



Scheme 1.4 – Plausible Mechanism for the Synthesis of the Tirandamycin Bicycle[17]

Lastly, following release from the terminal module, pre-tirandamycin is converted to TirC via a post-assembly line elimination reaction catalyzed by the TrdE glycoside hydrolase (Scheme 1.2).[21] This likely occurs via the transient breakdown of the tirandamycin bicycle to form an acidified oxonium intermediate, which subsequently is dehydrated by carboxylate containing residues within the active site (Scheme 1.5).



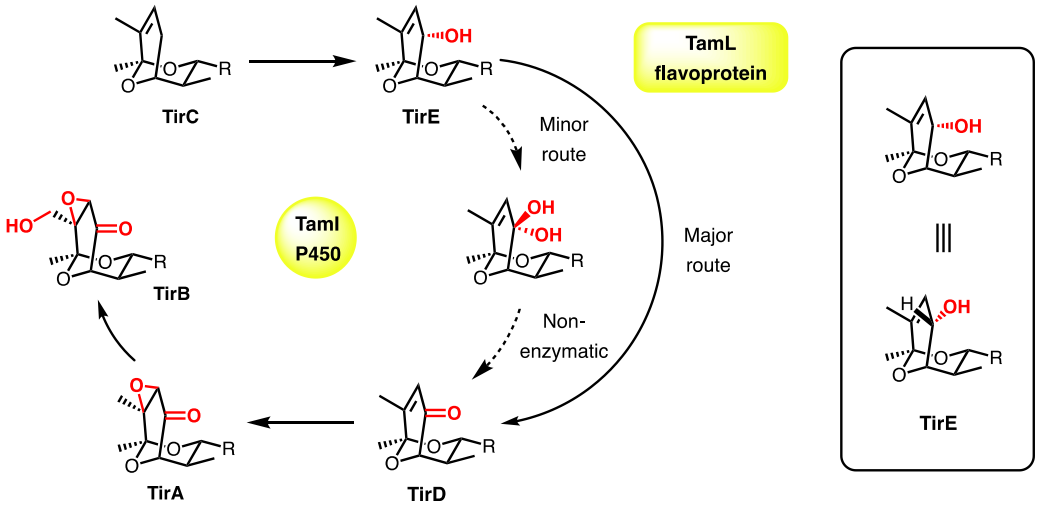
Scheme 1.5 – Proposed Mechanism for C10-C11 Double Bond Formation

1.4.2 Oxidative Tailoring of Tirandamycin

The final steps in the biosynthesis of tirandamycin involve the interplay of two co-dependent enzymes, the cytochrome P450 TamI and TamL flavoprotein, which are responsible for the oxidative modification of the tirandamycin bicycle (Scheme 1.6).[15] While cytochrome P450 monooxygenases are well known for enabling a variety of transformations[38], TamI is unique in that it is a versatile P450 which acts in tandem with another enzyme to install multiple functionality within a single substrate, highlighting it's remarkable flexibility in substrate recognition and catalysis.

TamI has been shown to promote three of four sequential steps in the oxidative cascade of tirandamycin. TirC is first oxidized by TamI at the C10 secondary allylic

position to give TirE, as discovered through the initial isolation of TirE by Sherman and co-workers.[15] While TirE formation was observed following incubation of TirC with recombinant P450 enzyme, only the latter accumulated from *tamI* disruption strains.[16]

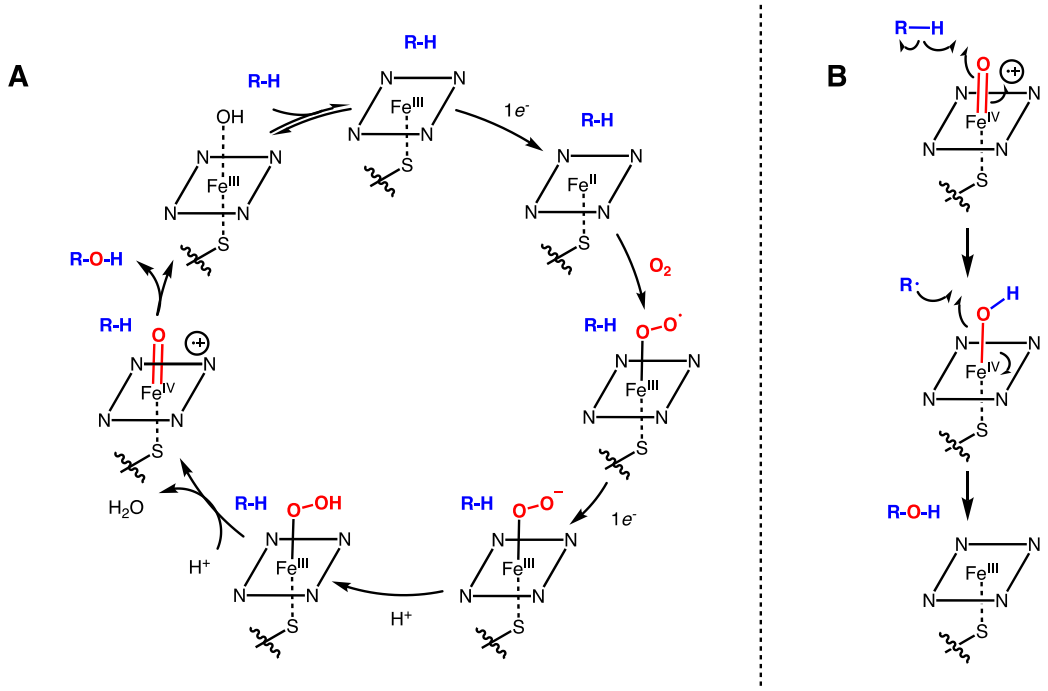


Scheme 1.6 – Oxidative Cascade of Tirandamycin in *Strep.* sp. 307-9

The mechanism of hydrocarbon hydroxylation by P450s is well studied in the literature.[38,39] Formation of the active catalyst involves incorporation of molecular oxygen to generate a porphyrin radical cation Fe(IV) oxo species, which is facilitated by the addition of two single electron reducing equivalents provided by NAD(P)H (Scheme 1.7A). A proximal hydrogen atom is then abstracted from the substrate, followed by rapid recombination of the alkyl radical via a radical rebound mechanism to give the hydroxylated product (Scheme 1.7B).

The minimal accumulation of downstream oxidation products from the incubation of TirC with TamI illustrates that while TamI is capable of catalyzing this process *in vitro*, another enzyme is likely responsible for the predominant formation of TirD.[15] Indeed, TamL was found to be effective at converting TirE to TirD, likely through reduction of a

covalently bound FAD cofactor. Crystal structures revealed tirandamycin binds in the active site of TamL with the bicyclic ketal moiety positioned towards FAD and the tetramic acid extended towards the mouth of the substrate binding cleft (Figure 1.5). Proximity of the C10 hydrogen towards N5 of the isoalloxazine ring is consistent with a hydride transfer mechanism, in which the C10 hydroxyl of tirandamycin is first deprotonated by a highly conserved Y447 residue. Y136 may also participate in this proton transfer network to help facilitate proton abstraction through activation of Y447.



Scheme 1.7 – Mechanism of P450 Catalyzed Hydroxylation **A:** Cytochrome P450 Catalytic Cycle **B:** H Atom Abstraction / Radical Rebound Mechanism

Following transfer of the tirandamycin substrate back to TamI, the C11-C12 olefin is epoxidized to form TirA, highlighting the diverse reactivity of this multifunctional P450 (Scheme 1.6). TirA can then be hydroxylated by TamI at the primary C18 methyl group, albeit with much greater inefficiency than previous TamI catalyzed transformations

(k_{cat}/K_m = 5.8 x 10^{-4} μM^{-1} min^{-1} as compared to 19.3 and 3.6 μM^{-1} min^{-1} for hydroxylation of TirC and epoxidation of TirD, respectively).[15] *In vitro* enzymatic conversions of TirD and TirA with recombinant TamI provide evidence for these final two steps in tirandamycin biosynthesis.

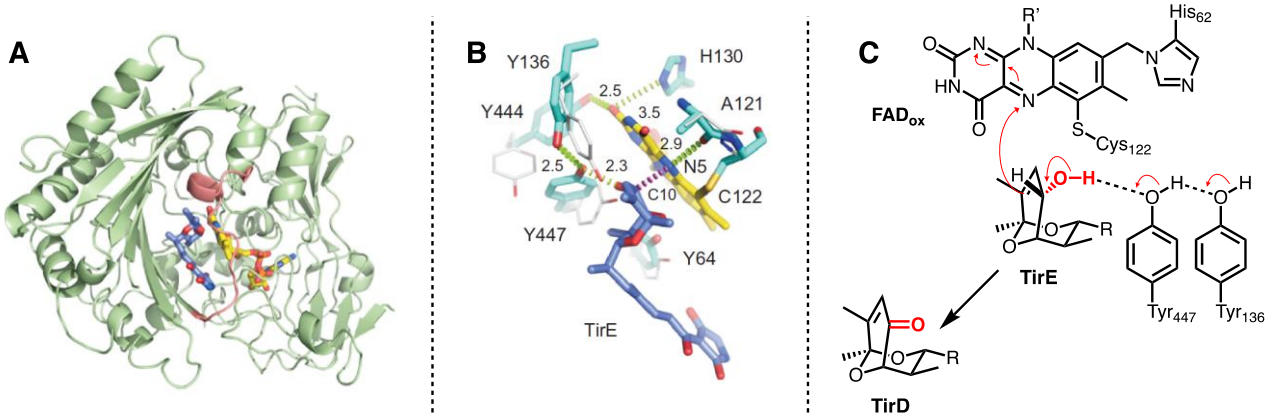
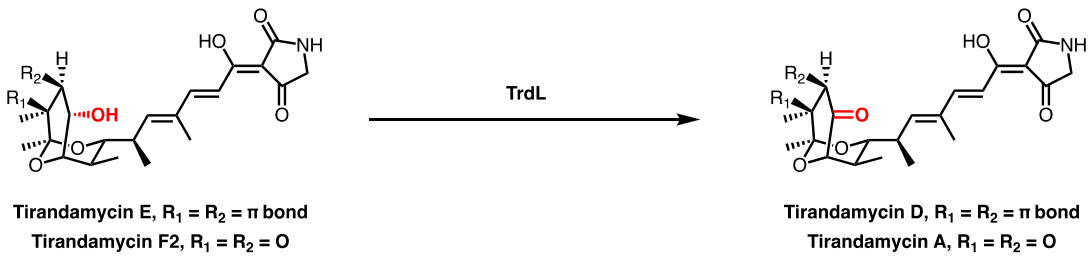


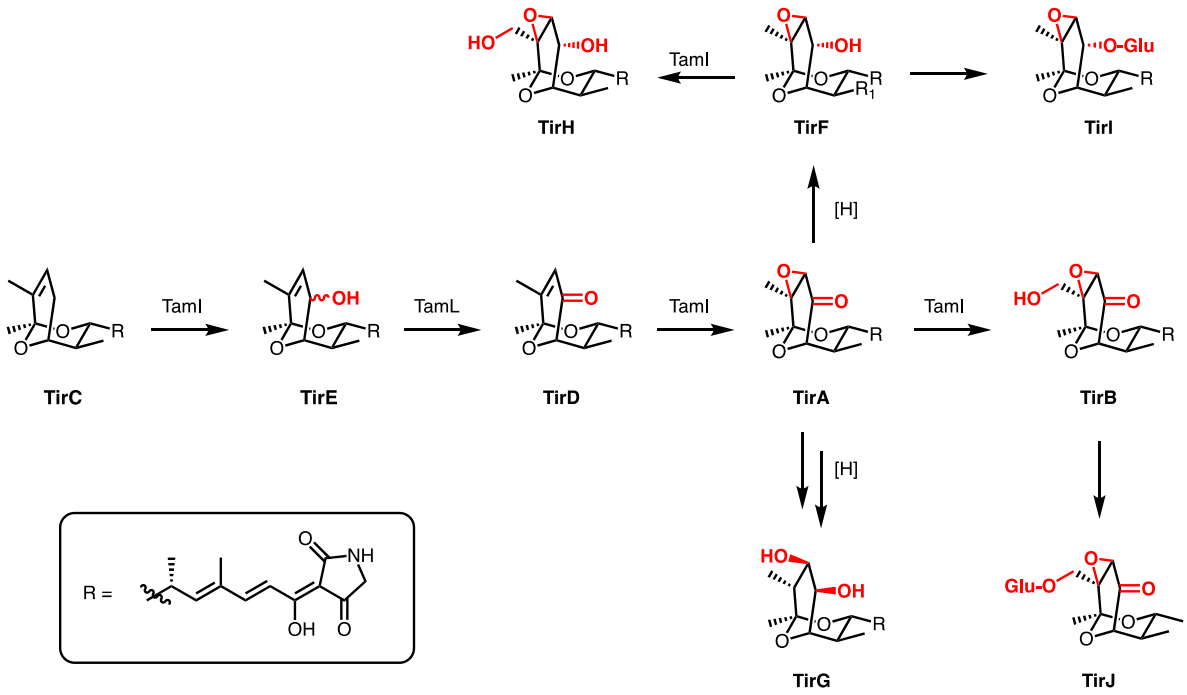
Figure 1.5 – TamL Crystal Structure **A**: Substrate-bound TamL Co-Crystal Structure (tirandamycin = blue sticks, FAD = yellow sticks). **B**: Superimposition of Substrate-Bound (cyan sticks) and Substrate-Free TamL (grey sticks) **C**: Mechanism of Dehydrogenation of TirE by TamL[15]

In *Strep.* sp. SCSIO1666 the flavin-dependent oxidoreductase TrdL was found to not only be responsible for the oxidation of TirE, but also the conversion of TirF2 to TirA (Scheme 1.8).[20] This led to the proposal of an alternative biosynthetic pathway in which TirA is accessed through epoxidation of TirE to give TirF2 followed by subsequent TrdL-catalyzed dehydrogenation.



Scheme 1.8 – C10 Dehydrogenation Catalyzed by TrdL Flavoprotein from *Strep.* sp. SCSIO1666

Shen has also suggested a more complex, divergent oxidative tailoring pathway which takes into consideration the isolation of other highly modified tirandamycin congeners from *Strep.* sp. 17944 (Scheme 1.9).[23] Both TirF and TirG, for example, could be formed through reduction of the C10 ketone of TirA. Furthermore, consecutive hydroxylation of TirF by TamI accounts for the exclusive isolation of TirH when TirF is produced in high yields. It is not clear, however, how TirI and TirJ are formed since no gene-encoding glucosyltransferase has been identified within the tirandamycin biosynthetic cluster, although Shen speculates that glycosylation of TirF and TirB may confer self-resistance to the bacterial organism. Additional experimental work is needed in order to answer these remaining questions and validate this proposed biosynthetic pathway.



Scheme 1.9 – Proposed Biosynthetic Pathway in *Strep.* sp. 17944

While significant work has been done to elucidate the role of TamI in the biosynthesis of tirandamycin, the underlying factors that contribute to the unique reactivity of multifunctional P450s are still not well understood.[40] The following two chapters will discuss efforts towards the synthesis of a variety of analogs of tirandamycin and their use in evaluating the mechanism of substrate binding and catalysis in TamI. These insights will not only lead to a greater appreciation of the functional diversity of cytochrome P450 enzymes in nature, but will be applied towards the development of P450 monooxygenases as versatile biocatalysts for C-H activation.

CHAPTER II

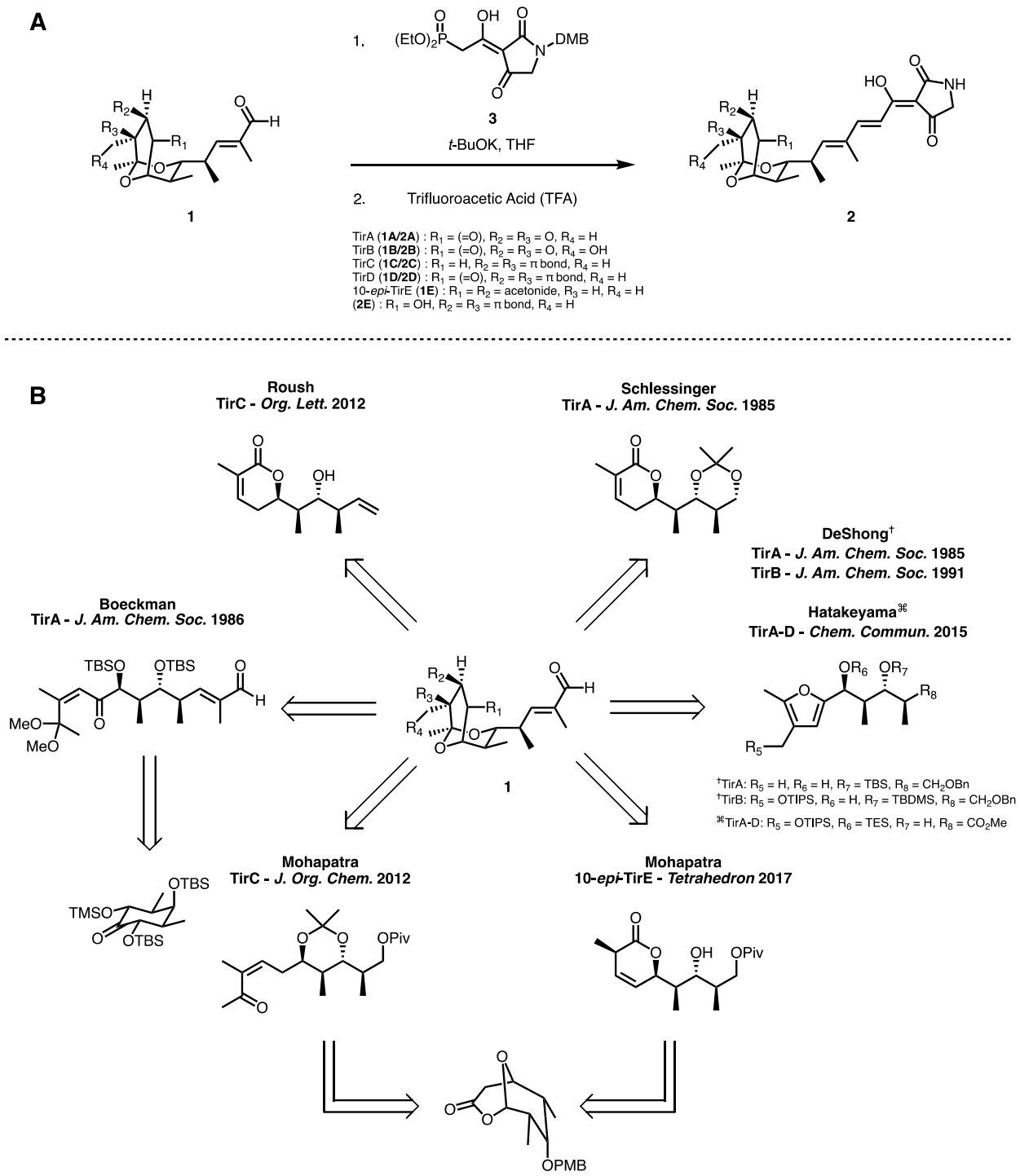
Synthesis of Tirandamycin & its Analogs

2.1 Total Synthesis of Tirandamycin

2.1.1 Overview of Synthetic Strategies Towards Tirandamycin

The unique structural features and biological activity[14,22,23,25,26,35] of tirandamycin make this natural product an intriguing synthetic target. It is therefore not surprising that tirandamycin and other related dienoyl tetramic acids have been the focus of a variety of synthetic efforts since the 1980s. Over ten different formal and total syntheses have been reported in the literature, the majority of which target TirA[41–48], the most potent congener of tirandamycin.[14] Nonetheless, more recent work has focused on the synthesis of other members of this natural product family, including TirB,[49,50] TirC,[51,52] TirD[50] and 10-*epi*-TirE.[53]

Despite the variety of different approaches towards the construction of tirandamycin, many of these reported methods share a number of similarities. Most notably, almost all of these syntheses feature a common enal intermediate (**1**), which allows for the late stage attachment of the 3-acyl tetramic acid moiety (Scheme 2.1). Additionally, these synthetic strategies share several complementary retrosynthetic disconnections, including assembly of the tirandamycin bicycle through oxidation of a substituted tetrahydrofuran ring (DeShong[42,49] and Hatakeyama[50]), methyl lithium

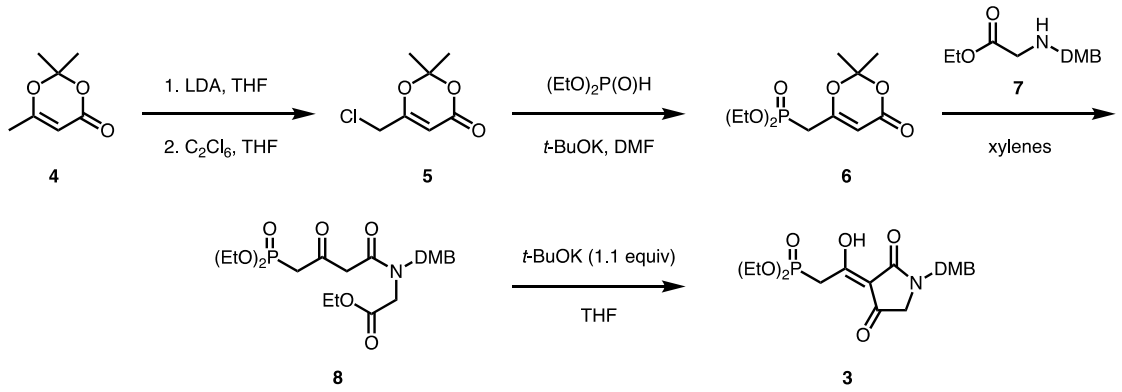


Scheme 2.1 – Synthesis and Attachment of a Common Late Stage Intermediate in the Total Synthesis of Tirandamycin **A**: Attachment of Tetramic Acid via Horner-Wadsworth Emmons Olefination **B**: Overview of Various Synthetic Approaches Towards a Common Late Stage Intermediate

addition followed by subsequent ketalization of a lactone intermediate (Schlessinger[41] and Roush[51]), and use of a desymmetrization technique to access the synthetically challenging *anti, anti*-dipropionate stereotriad unit (Boeckman[44] and Mohapatra[52,53]).

In the penultimate step of tirandamycin synthesis, coupling of a readily accessible phosphonate tetramic acid reagent (**3**) via Horner-Wadsworth-Emmons (HWE) olefination affords 2,4-dimethoxybenzyl (DMB) protected tirandamycin (Scheme 2.1A). Introduction of the benzyl substituent was found to be necessary for successful conversion to product, as the aldehyde was otherwise unstable under the strongly basic conditions necessary for condensation of the unprotected tetramic acid.[41,42,44] Cleavage of the DMB group is then achieved through brief treatment with trifluoroacetic acid (TFA) to give the target compound.

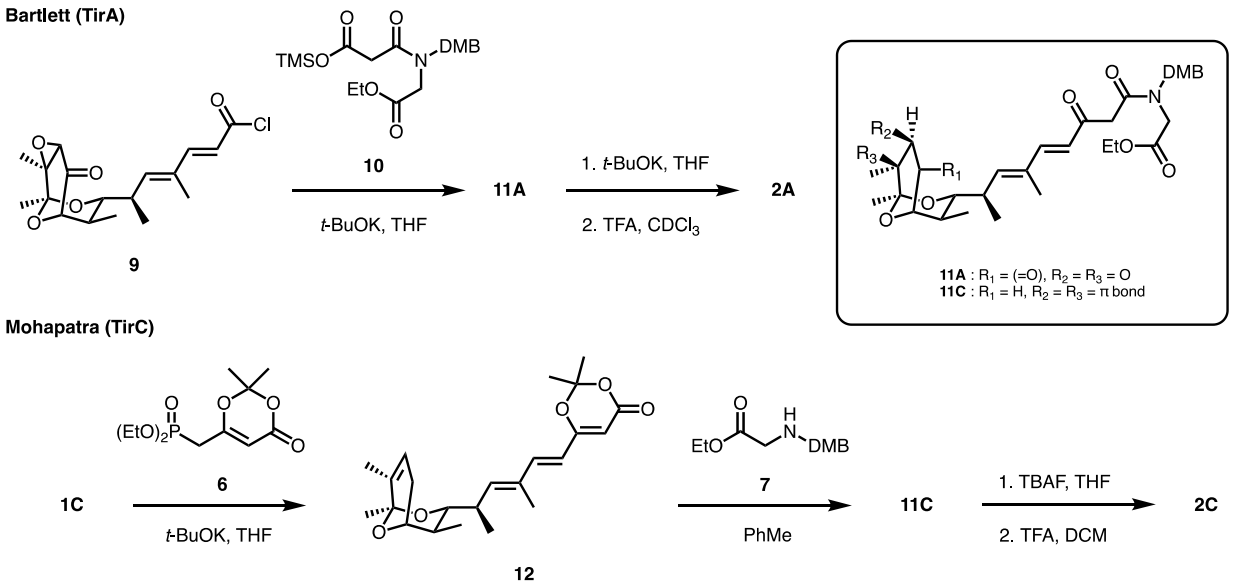
As initially reported by Boeckman[44,54] and Schlessinger,[41] the requisite phosphonate tetramic acid **3** can be obtained in five steps from 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (**4**, Scheme 2.2). Allylic chlorination followed by displacement with diethyl phosphite provides dioxolenone phosphonate **6**,[55] which upon treatment with DMB glycine ester **7** results in the formation of β keto amide **8**. Lastly, Dieckmann condensation of **8** upon exposure to *t*-BuOK gives the desired HWE tetramic acid.



Scheme 2.2 – Synthesis of Phosphonate Tetramic Acid **3**

While incorporation of the tetramic acid moiety usually involves direct coupling of the phosphonate tetramic acid (**3**, Scheme 2.1A), in a few cases cyclization of the tetramic acid ring occurs following its initial attachment to the tirandamycin bicycle (Scheme 2.3). For example, acylation of a silyl malonamidate derivative by Bartlett et al.[45] leads to β-ketoamide **11**, which is then cyclized and deprotected in a manner similar to Boeckman[44] and Schlessinger.[41] On the other hand, the total synthesis of TirC by Mohapatra and co-workers[52] involves HWE olefination of phosphonate **6** to ultimately furnish the DMB-protected natural product.

The remainder of this chapter will include a brief description of the first synthetic routes to TirA by DeShong[42] and Schlessinger,[41] followed by an account of a more recent approach to TirC.[51] This latter work by Roush motivated our efforts towards an abbreviated synthesis of various analogs of the tirandamycin bicycle, which were subsequently used as unnatural substrates in a variety of bioenzymatic reactions (Chapter 3).



Scheme 2.3 – Alternative Methods for Tetramic Acid Incorporation

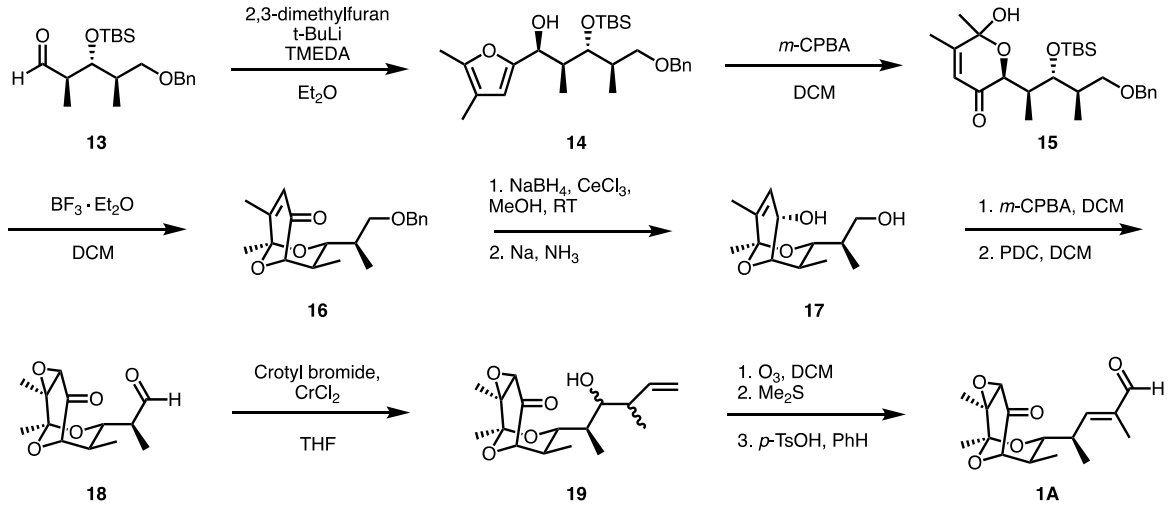
2.1.2 Total Synthesis of TirA by DeShong and Schlessinger

Two separate accounts on the total synthesis of TirA were reported concurrently in 1985 by the DeShong[42] and Schlessinger[41] research groups, making them seminal efforts towards the synthesis of tirandamycin. Both publications featured two different approaches en route to late stage intermediate **1A**, each of which has influenced the development of later syntheses of this class of natural products (Scheme 2.1).

The synthesis reported by DeShong et al. affords racemic TirA in 12 steps from simple starting materials (Scheme 2.4).[42] Lithiation of 2,3-dimethylfuran followed by condensation with aldehyde **13**[56] gave a 1:1 mixture of diastereomers which were separated by column chromatography. The desired stereoisomer, **14**, was then oxidized with *m*-CPBA to yield pyranone **15**, a key intermediate towards construction of the 2,9-dioxabicyclononane ring system. BF_3-Et_2O was found to readily catalyze the

successive removal of the silyl ether protecting group from **15**, inducing cyclization to the bicyclic ketal.

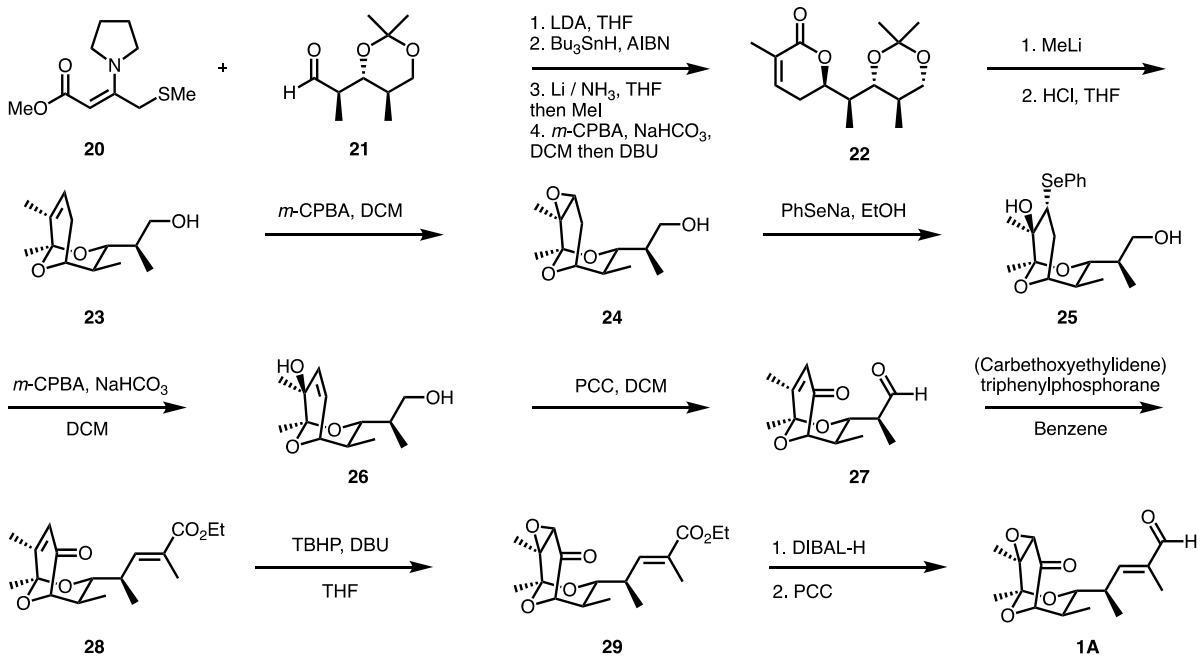
The next several steps of DeShong's synthesis focus on incorporation of the requisite epoxide. Since direct epoxidation of the electron deficient olefin was low yielding, a circuitous route involving $NaBH_4$ reduction of the enone followed by subsequent benzyl ether removal was pursued instead. Luckily, *m*-CPBA-catalyzed epoxidation of the resulting diol (**17**) proceeded smoothly with stereoselective addition to the *exo* face of the bicycle. Homologation of the corresponding aldehyde (**18**), followed by ozonolysis and dehydration then provide **1A** as the penultimate precursor to tirandamycin. Incorporation of the tetramic acid moiety was accomplished following the previously noted conditions shown in Scheme 2.1A.



Scheme 2.4 – DeShong's Route to **1A** as Part of the Total Synthesis of (±) – TirA

Schlessenger's complementary approach towards the synthesis of enantiopure TirA requires 16 steps to access the final product via the intermediacy of acetonide **22** (Scheme 2.5).[41] Anti-selective aldol condensation of a lithium enolate derived from

vinylogous urethane **20** affords the desired substituted lactone,[57] which upon exposure to Bu_3SnH and AIBN, results in the removal of the stereo-defining thiomethyl adduct. Reductive methylation followed by consecutive removal of the pyrrolidine residue with *m*-CPBA gives the target intermediate (**22**).



Scheme 2.5 – Schlessinger's Route to **1A** as Part of the Total Synthesis of (–) – TirA

One highlight of Schlessinger's synthesis is the remarkably simple yet efficient conversion of **22** to **23** through MeLi addition and concomitant acid catalyzed cyclization to furnish the bicylic ketal. Unfortunately, attempts to oxidize **23** to aldehyde enone **27** were not as straightforward, as treatment with chromium trioxide 3,5-dimethylpyrazole led to an erosion in stereochemistry in the final product. A more indirect route from **23** to **27** was therefore utilized instead.

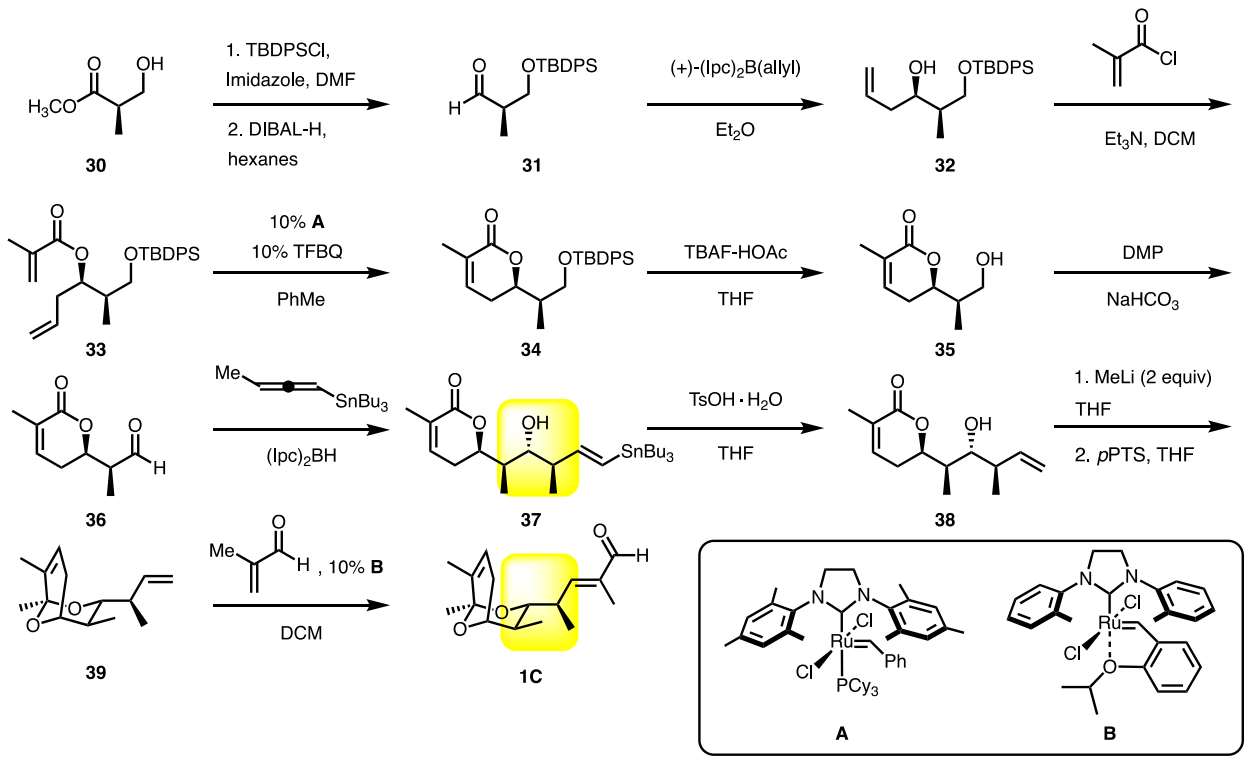
Lastly, in the first of a final series of transformations, Wittig olefination with (carbethoxyethylidene)triphenylphosphorane was successful in helping extend the side

chain towards formation of the 1-oxopentadienyl tether. Efficient introduction of the epoxide was then achieved under nucleophilic conditions (*t*-Butyl hydroperoxide/DBU) to give **29**, illustrating an improvement upon DeShong's multi-step approach.[42] Following a few minor functional group interconversions, synthesis of (–) – TirA was completed as described earlier from **1A** (Scheme 2.1A).

2.1.3 Total Synthesis of TirC by Roush

Many years later, the Roush group identified tirandamycin as an appropriate synthetic target to demonstrate the utility of their own chiral crotyl-borylating reagent in the context of a more complex natural product system.[51,58] Specifically, Roush and co-workers recognized the *anti, anti*-dipropionate stereotriad unit present in tirandamycin (highlighted in yellow in Scheme 2.6) as an accessible motif through mismatched double asymmetric γ-stannylcrotyl boration of intermediate **36**. The resulting stannyl homoallylic alcohol **37**, which was obtained with >15:1 stereoselectivity, provided direct access to (–) – TirC in another six steps, thereby completing the first total synthesis of this particular tirandamycin congener. Notably, MeLi addition to lactone intermediate **38** followed by successive exposure to catalytic acid borrows from methodology previously reported by Schlessinger[41] to provide the bicyclic ketal.

As described in the next section, the synthesis of TirC by Roush has strongly influenced our work towards the engineering of a collection of enzymatic substrates modeled after this class of natural products.



Scheme 2.6 – Roush's Route to **1C** as Part of the Total Synthesis of (–) – TirC

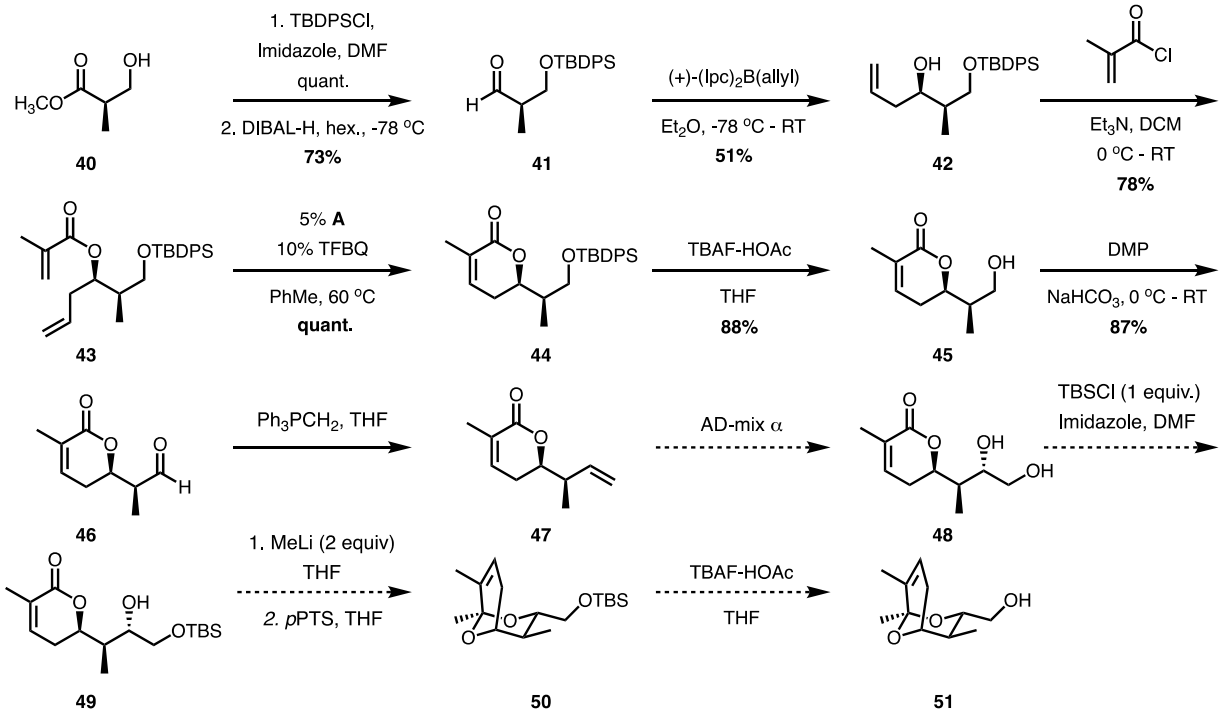
2.2 Synthesis of Tirandamycin Analogs

2.2.1 Initial Approach Towards Synthesis of TirC Bicycle

Encouraged by precedent in the literature on the synthesis of tirandamycin, we set out to create a series of tirandamycin analogs to help probe the effect of substrate structure on the multifunctional activity of P450s such as TamI. Furthermore, access to a variety of unnatural tirandamycin analogs creates a critical opportunity to allow for the continued exploration of the structure-activity relationship of this important class of bioactive molecules.[14,22,23,25,26,35]

We envisioned these analogs to closely resemble the bicycle of tirandamycin, the only structural feature to be oxidized by TamI as part of the tirandamycin biosynthetic pathway[15] and a necessary structural feature influencing its pharmacological

activity.[15,17] These analogs also required a functional handle to allow for attachment of a synthetic “anchor” which would facilitate binding within the enzyme active site (see chapter 3). Considering that TirC is well understood to precede P450 catalyzed oxidative tailoring,[15] our initial plan was to synthesize the bicycle of TirC with a free hydroxyl located off of C6 in place of the naturally occurring dienoyl side chain (**51**, Scheme 2.7). This alcohol would serve as a point of attachment for carboxylate containing anchors or could otherwise be easily modified to allow for incorporation of other anchoring functionalities.



Scheme 2.7 – Initial Approach Towards an Analog of TirC

Our original intent was not to reinvent the synthesis of the TirC bicycle but to instead utilize the methods previously established by Roush[51] (Scheme 2.6) to access analog **51** as quickly as possible. Starting from the commercially available ester (**40)**, TBDPS protection followed by DIBAL-H reduction of the resulting ester gave aldehyde

41 in a 73% yield over two steps. Allylboration using an in situ generated (+)-$(Ipc)_2$ allylborane species provided enantioenriched homoallylic alcohol **42** in moderate yield (51%). Acylation of **42** with methacryloyl chloride then gave ring closing metathesis (RCM) precursor **43** in 78% yield.

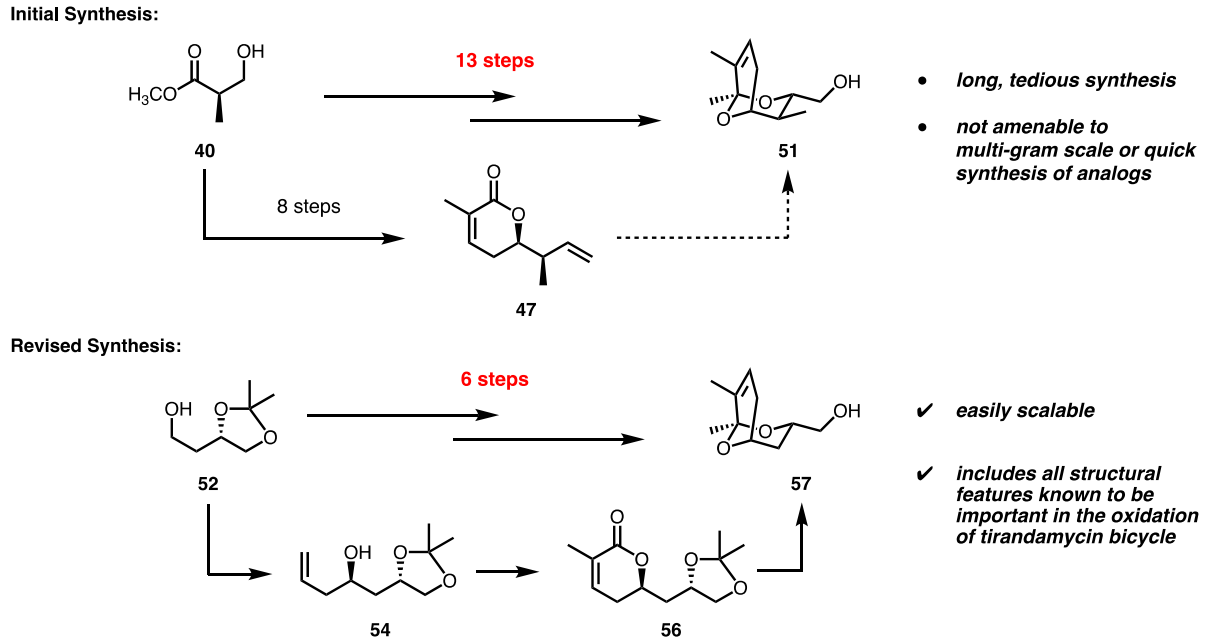
Ring closing metathesis conditions were initially chosen according to the procedure by Roush et al.,[51] which led to low conversions (≤37%). Sparging the system drove the reaction forward by driving off ethylene byproduct, providing quantitative yields in less than two hours with decreased catalyst loading (5 mol%). Tetrafluoro-1,4-benzoquinone was added to prevent unwanted isomerization caused by ruthenium hydride species present in solution.[59]

Following ring closure, lactone **44** was deprotected using TBAF in an acetic acid buffer to give **45** in 88% yield. Oxidation of the resulting alcohol using Dess-Martin periodinane (DMP) provided aldehyde **46** in 87% yield. **46** was then treated with methylene triphenylphosphine under suitable Wittig conditions to afford trace amounts of the corresponding olefin.

2.2.2 Simplified Synthesis of TirC Bicycle

At this point in our work we re-evaluated the practicality of achieving our goal of quickly obtaining **51** in sufficient quantities for rapid diversification and enzymatic screening. Rather than repeating previous work to bring forward more material and carry forward our initial strategy, we recognized that this approach was not amenable to efficient, large-scale substrate synthesis. By eliminating incorporation of the C17 methyl group, we realized that an otherwise analogous TirC analog (**57**) could be accessed in 6 steps instead of 13 steps (Scheme 2.8). Since C17 is remote from the multiple sites of

oxidation in the biosynthesis of tirandamycin and is instead located proximal to the point of anchor attachment, (a region typically not oxidized by P450s which operate via an anchoring type mechanism), its omittance is likely to be inconsequential in our analysis.



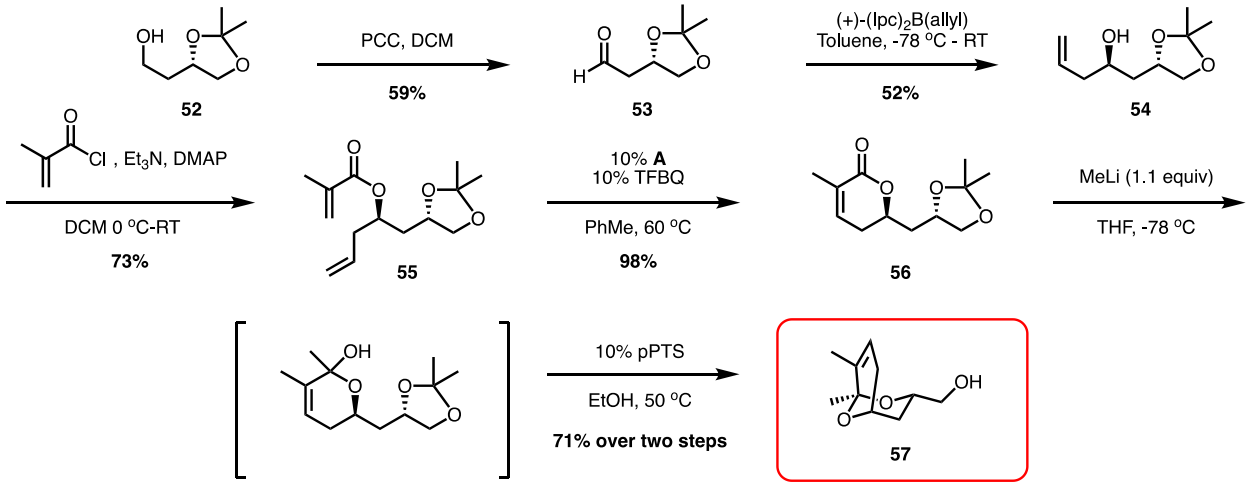
Scheme 2.8 – Comparison of Synthetic Strategies Towards a TirC Analog

Altering our focus towards a more simplified approach fulfills a noticeable gap in the literature in this area. While prior work on the synthesis of tirandamycin is extensive, no methods exist for the direct access to the tirandamycin core with minimal synthetic manipulation. The discovery of a more viable route towards tirandamycin would therefore enable more rapid pharmacological evaluation of this family of natural products.

Utilization of (4*S*)-(+)-4-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxolane (**52**) as the starting material for our second generation synthesis strategically provides the C7 stereocenter, which often is installed through more complex asymmetric methods[51]

(Scheme 2.9). **52** Is currently available for $12/g from Sigma Aldrich,[60] making it a cost-effective reagent for large-scale synthesis.

PCC oxidation of **52** afforded the requisite aldehyde, albeit in diminished yields (59%) due to the volatility of **53**. Adaptation of methods previously established by Roush, which were also utilized in our previous synthesis, then provided homoallylic alcohol **54** in 52% yield after being purified twice by column chromatography. Residual crude product was typically set aside and purified with product mixtures from later reactions to isolate remaining material. Acylation of **54** with methacryloyl chloride subsequently gave ester **55** in 73% yield, which was aided by the addition of DMAP to the reaction.



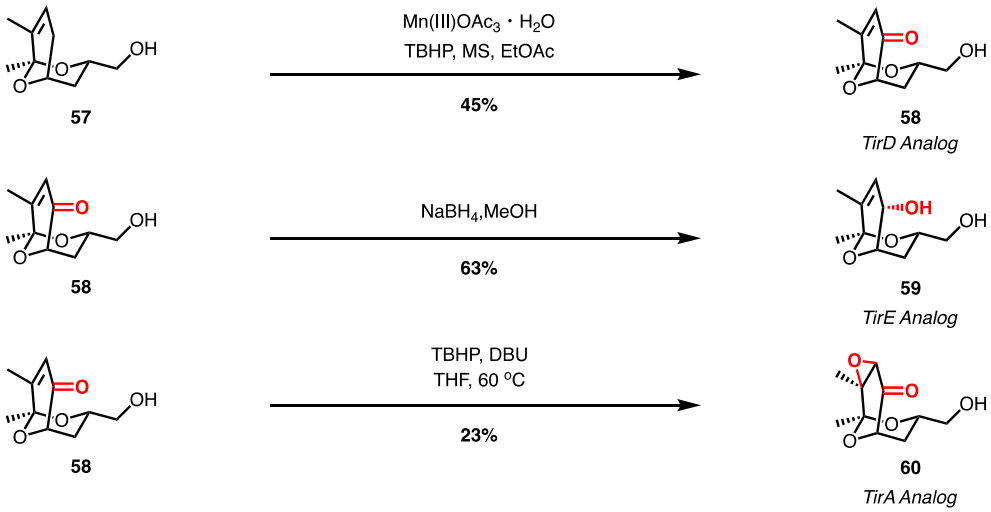
Scheme 2.9 – Improved Synthesis of an Analog of TirC

Use of the previously established RCM conditions from the original analog synthesis did not work well on a larger scale (> 0.8 mmol), resulting in significant dimer formation and incomplete conversion of starting material. Fortunately, addition of an extra 5 mol% of catalyst several hours after the initial catalyst addition drove the

reaction to full conversion to give product **56** in near quantitative yields (98%). Lastly, MeLi addition followed by tandem acetonide deprotection and cyclization produced TirC analog **57** in a 71% yield over the final two steps.

2.2.3 Diversification of Analog Synthesis

In order to further assess the impact of substrate structure on P450 activity, we explored our ability to use late stage chemical oxidation methods as a means towards a wider variety of tirandamycin analogs beyond TirC. Gratifyingly, manganese (III) acetate catalyzed allylic oxidation[61] of **57** provided enone **58**, a TirD analog, in moderate yields (45%, Scheme 2.10). Stereoselective reduction of the allylic ketone, in which the hydride was introduced exclusively onto the exo face, gave TirE analog **59** in one additional step (63% yield).



Scheme 2.10 – Synthesis of Additional Analogs of Tirandamycin

We were also pleased to find that higher oxidation analogs of tirandamycin could be synthesized through selection of the appropriate reaction conditions. Although the

use of electrophilic oxidants such as *m*-CPBA were unreactive with the electron deficient olefin of **58**, treatment with *t*-Butyl hydroperoxide and DBU afforded TirA analog **60** with good facial selectivity.

2.3 Conclusions and Future Directions

In summary, we have reported a concise route towards the synthesis of a series of tirandamycin analogs which greatly improves upon the utility of previous synthetic approaches. Future work will include the application of new chemical methods to afford key intermediates in a more novel and efficient manner. The increased accessibility of this scaffold not only enables research on tirandamycin as a substrate for P450s (chapter 3), but opens the door for future structure-activity studies on this important class of biomolecules. Results from this chapter will be published in due course.

2.4 Collaborator Acknowledgements

Stanna Dorn (Hope College) and Sara Alektiar (University of Michigan) are both thanked for their help with tirandamycin analog synthesis. Stanna initially synthesized compound **58**.

CHAPTER III

Strategies for Enabling P450 Oxidations & Applications to the Tirandamycin Core

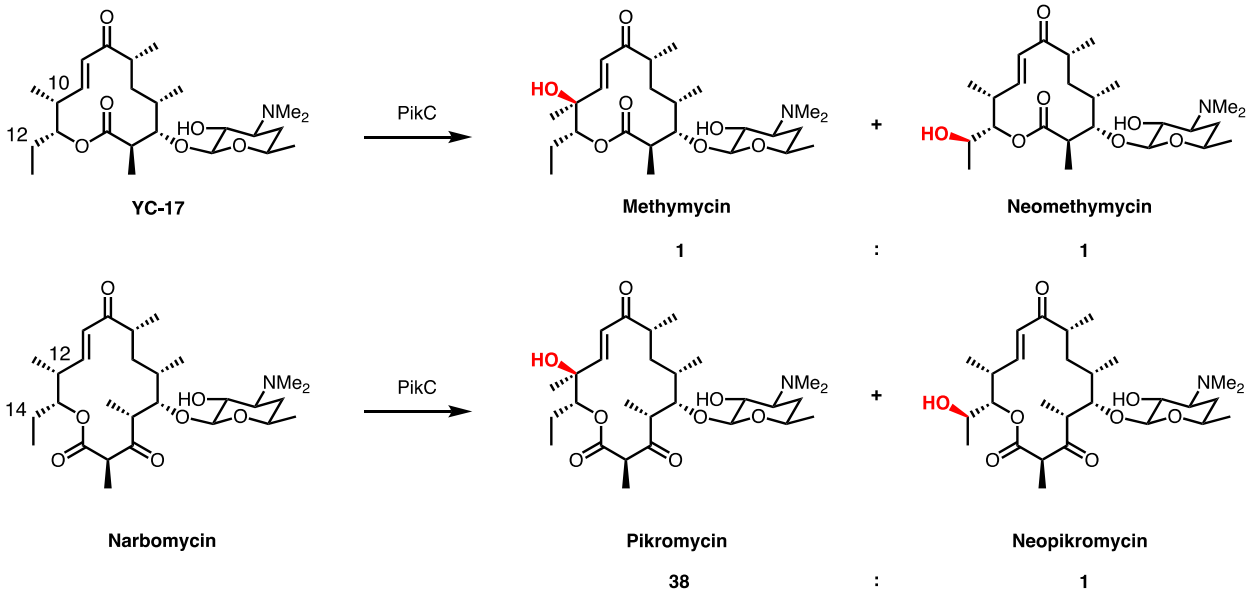
Our initial interest in tirandamycin and the cytochrome P450 TamI stems from the Montgomery and Sherman groups' long-standing history in P450 mediated biocatalysis. Utilization of the engineered P450 PikC has resulted in several substantial contributions to the field, such as the regioselective hydroxylation of macrocyclic[62] and small molecule ring systems,[63] including the tirandamycin core. The following chapter will discuss our successful implementation of a substrate engineering approach[64,65] with PikC and early work towards development of a similar strategy with TamI to address some of the limitations of these previous methods.

3.1 Development of PikC as a General Catalyst for C-H Functionalization

3.1.1 PikC Mediated Oxidation of Macrolide Antibiotics

The bacterial P450 monooxygenase PikC is responsible for the oxidative tailoring of the 12- and 14-membered macrolides YC-17 and narbomycin in *Strep. venezuelae* (Scheme 3.1).[66] YC-17 and narbomycin are produced from variable termination of polyketide chain elongation intermediates in the pikromycin biosynthetic pathway, followed by DesVII catalyzed attachment of desosamine.[67,68] Desosamine has been found to affect both the biological activity of macrolide antibiotics[69–71] and their post-PKS modification[72] through a unique anchoring mechanism involving the *N,N*-dimethylamino

group of the sugar. Salt bridge interactions between the protonated dimethylamino group of YC-17 and narbomycin play a key role in substrate binding and catalysis with PikC, affording methymycin and neomethymycin in 1:1 ratio and pikromycin over neopikromycin in a 38:1 ratio (Scheme 3.1). Dihydroxylated macrolide novamethymycin has also been isolated and characterized as a minor oxidation product of the PikC catalyzed reaction in *Strep. venezuelae*.[73]



Scheme 3.1 – PikC Catalyzed Site Selective Oxidation of Macrolide Antibiotics YC-17 and Narbomycin

YC-17 and narbomycin are anchored in two distinct binding pockets of PikC, as shown by co-crystal structures of both substrates in the enzyme active site (Figure 3.1).[72] While narbomycin is anchored by salt bridge contact with Glu-85 in the exposed pocket, YC-17 is positioned in the buried pocket by interaction with Glu-94, bringing C10 and C12 of the macrolactone ring within close proximity of the heme iron for regioselective oxidation. Elimination of the surface exposed Asp-50 residue results in

the formation of a more active $PikC_{D50N}$ mutant by relocation of substrate to the catalytically productive buried site.[74]

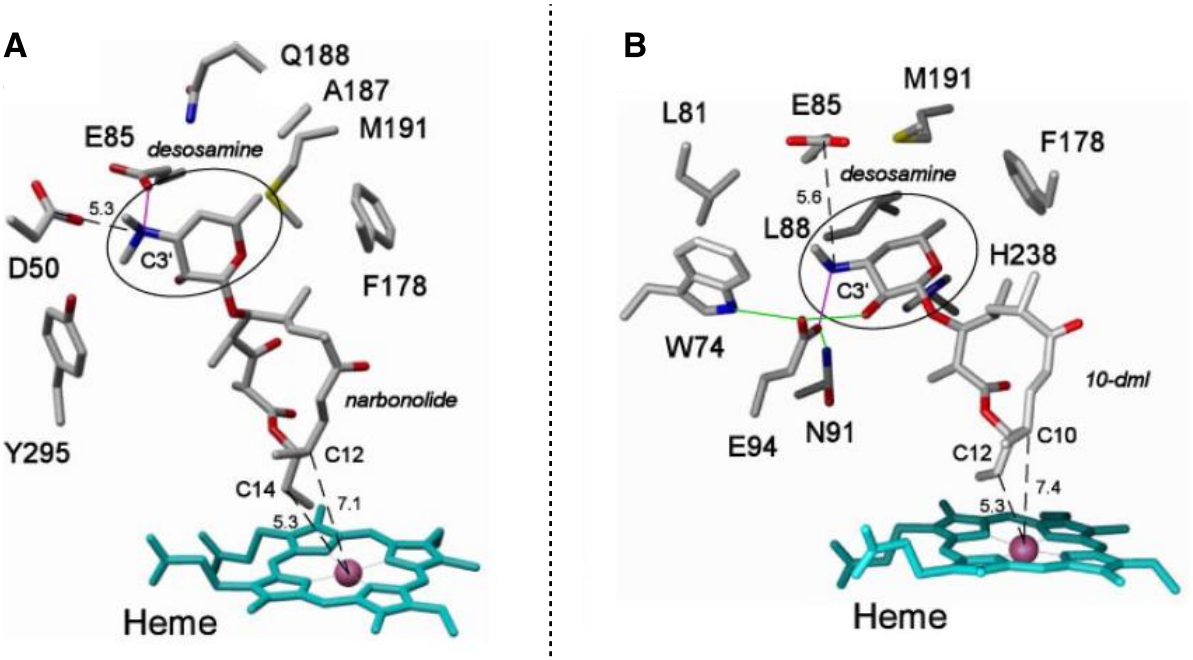


Figure 3.1 – Co-crystal Structure Showing Desosamine Binding Interactions of Narbomycin (**A**) and YC-17 (**B**) in the PikC Active Site (*Hydrogen bonds are shown in green, salt bridges are in magenta and distances are in Angstroms*)[72]

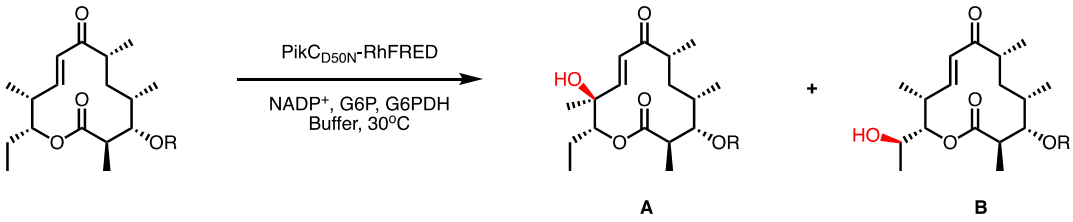
The remarkable flexibility of PikC is shown through its accommodation of endogenous substrates of different ring sizes and functionalization patterns. This is largely due to the non-specific hydrophobic interactions that bind both macrolactone rings in YC-17 and narbomycin,[72] allowing for its potential exploitation as a biocatalyst for a broad range of substrate classes. The remainder of this section will describe our efforts towards the use of synthetic dimethylamino anchoring groups to control the regioselectivity of PikC catalyzed C-H bond functionalization and access new analogs of tirandamycin which do not resemble readily observable biosynthetic pathway intermediates.

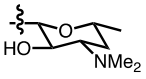
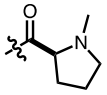
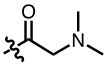
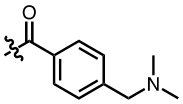
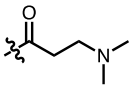
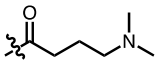
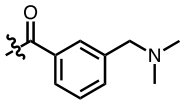
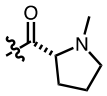
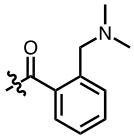
3.1.2 Directing Group-Controlled Regioselective Oxidation of YC-17 Analogs

Early studies by the Sherman and Montgomery groups validated the use of PikC as a general catalyst for the oxidation of a series of carbocyclic ring systems appended to a desosamine sugar anchor.[75] While desosamine was able to promote reactivity of unnatural substrates with PikC, difficulties associated with the synthesis and removal of desosamine limit its widespread usage. We therefore proposed a series of readily accessible, simplified anchoring groups that would be able to mimic the binding capabilities of desosamine through incorporation of various tertiary-amine-containing functionalities. Appendage of these anchoring elements to the C3 hydroxyl of 10-deoxymethynolide (10-dml) resulted in an array of YC-17 analogs which were subsequently reacted with an engineered, self-sufficient fusion protein,[74,76] $PikC_{D50N}$-RhFRED (Table 3.1).[62] Use of an ester linkage allowed for ease in attachment via DCC coupling and subsequent hydrolysis of the directing group following enzymatic oxidation.

In all cases, PikC oxidized YC-17 analogs to give a mixture of two products resulting from hydroxylation at C10 and C12, as comparable to its observed reactivity in nature (Scheme 3.1). Interestingly, however, the replacement of desosamine with synthetic anchoring groups was found to alter the regioselectivity of the reaction based on the size, stereochemistry and rigidity of the chosen anchor. Among the three YC-17 analogs attached to linear anchors (**61**, **62**, and **63**), three-carbon propanoate derivative **62** gave the highest conversion and selectivity of monohydroxylated products (>99%, 1:3 in favor of C12 oxidation). Variation in enzymatic conversion was also reflected by the total turnover number (TTN, moles of product/moles of enzyme) for each of these

substrates, which was greatest for **62** (544). Notably, extension of the linker by one ethylene unit to give **63** reversed the regioselectivity from **61** and **62** in slight favor of allylic C10 oxygenation (1.8:1).



Compound	R	Product Ratio (A:B)	Conversion‡	TTN	Compound	R	Product Ratio (A:B)	Conversion‡	TTN
YC-17		1:1	—	896	65		1.8:1	94 (70%)	485
61		1:1.6	80% (46%)	260	66		10:1	> 99% (63%)	602
62		1:3	> 99% (73%)	544					
63		1.8:1	91% (89%)	452	67		> 20:1	> 99 (74%)	580
64		3:1	94 (77%)	456	68		1:4	77 (29%)	152

‡Isolated yield from preparative-scale reactions shown in parentheses.

Table 3.1 – Anchor Controlled Regioselectivity of YC-17 Analogs

In the case of *N*-methylproline derived substrates **64** and **65**, expansion to a more structurally rigid anchor lead to improvement in conversion and TTN in comparison to linear anchor **61**, which placed the amine a similar distance away from the macrolactone core. This structural modification was also found to have a significant impact on the selectivity of C-H bond oxygenation, showing preferential formation for methymycin derivative **A** over neomethymycin derivative **B** in up to a 3:1 ratio. The

greatest selectivity, however, was observed through my early work as a graduate student in the synthesis and attachment of various benzoic acid anchors to 10-dml. As shown in table 3.1, benzylic substrates **67** and **68** showed a dramatic difference in regioselectivity (>20:1 and 1:4, respectively), likely due to restriction of the conformational freedom of the substrate within the enzyme active site. This example of substrate controlled regioreversal further illustrates how simple structural changes can lead to substantial differences in reactivity, adding to chemists' understanding of how enzymes such as PikC can be used as catalysts for the functionalization of carbon-hydrogen bonds.

3.1.3 Using PikC as a Biocatalyst for the Oxidation of the Tirandamycin Bicycle

3.1.3.1 Site-Selective Oxidation of Simple Ring Systems

Soon after our initial discovery of the utility of synthetic anchors, co-workers within the Sherman and Montgomery laboratories demonstrated their application in the context of unnatural substrate classes (Figure 3.2).[63] (–)-Menthol (**69**) was chosen as a model substrate for anchor attachment due to its availability as a single enantiomer and its inclusion of a range of C-H bonds of variable bond strengths. Molecular dynamics (MD) simulations were used to rationally design a PikC triple mutant, $PikC_{D50ND176QE246A}$-RhFRED, which led to an increase in catalytic turnover caused by a shift in equilibrium towards a more active protein conformation. In addition to menthol, a variety of other ten carbon ring systems (**70-73**) were selectively oxidized by the engineered PikC triple mutant at sites distal to the point of attachment of the anchor, even in the presence of more activated C-H bonds.

Nevertheless, despite the remarkable flexibility of PikC, its capabilities have

remained limited primarily to the catalysis of mono-hydroxylation reactions. Even in the case of olefin-containing substrates such as **70** and **71**, PikC has never before been shown to exhibit multifunctional behavior comparable to other P450 monooxygenases such as TamI, raising questions as to the factors that distinguish between the reactivity of these two classes of enzymes. Given precedent for the large influence of substrate structure on P450 activity, it is possible that certain structural features present in the natural substrates of multifunctional P450s may play a role in their observed catalytic versatility. Our goal was therefore to use tirandamycin, the endogenous substrate of the multifunctional P450 TamI, as a substrate for PikC in order to investigate its potential mixed function capacity.

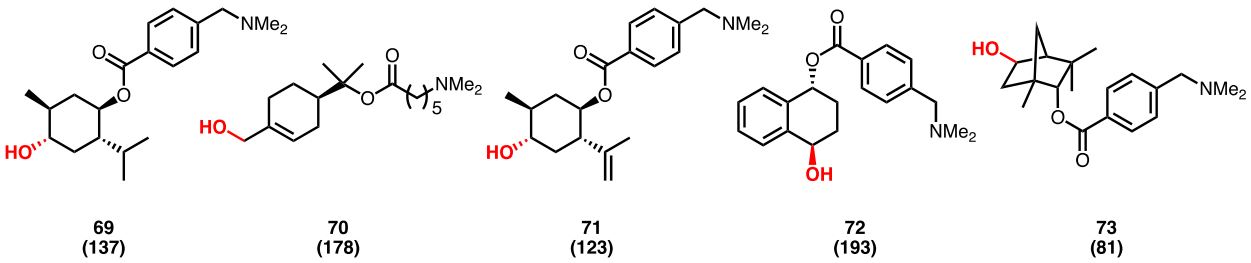
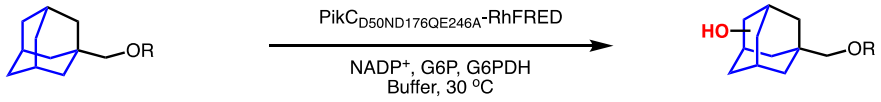


Figure 3.2 – Site Selective Oxidation of Small Molecule Ring Systems with PikC$_{D50ND176QE246A}$-RhFRED (*Numbers in parentheses are TTN for given substrate*)

Prior to the synthesis of a series of tirandamycin analogs (section 2.2), 1-adamantanemethanol was used as a model to test the feasibility of the tirandamycin bicycle to serve as a substrate for PikC (Table 3.2). In spite of its relative promiscuity, certain substrates are incompatible with PikC due to size and structural restraints.[75] Although it is not inconceivable that the tirandamycin bicycle would serve as a competent scaffold, this is difficult to predict without time-intensive computational modeling efforts. As an alternative, we decided to attach 1-adamantanemethanol to a

variety of PikC anchors, including more recently developed 1,4- and 1,5-triazole anchors (**79** and **80**) which were assembled from the corresponding azido acid and dimethyl amino containing alkyne.[77] While not a perfect model, both adamantane and the tirandamycin bicycle share a related skeletal framework (highlighted in blue in Table 3.2), which should be accommodated similarly within the PikC active site. Gratifyingly, modest to high conversions of adamantane derivatives with a range of linear and aryl anchors confirmed that the time intensive synthesis of the tirandamycin bicycle remained a worthwhile endeavor.



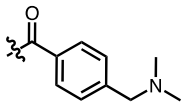
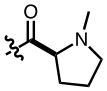
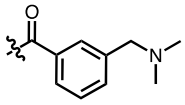
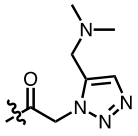
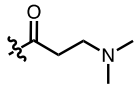
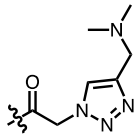
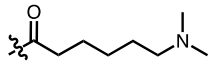
Compound	R	Enzymatic Conversion	Compound	R	Enzymatic Conversion
74		63% conversion to M+H+16 (1 major product)	**78**		36% conversion to M+H+16 (at least 3 products)
75		47% conversion to M+H+16 (1 major product)	**79**		Insignificant Conversion
76		19% conversion to M+H+16 (at least 3 products)	**80**		67% conversion to M+H+16 (at least 3 products)
77		75% conversion to M+H+16 (at least 3 products)			

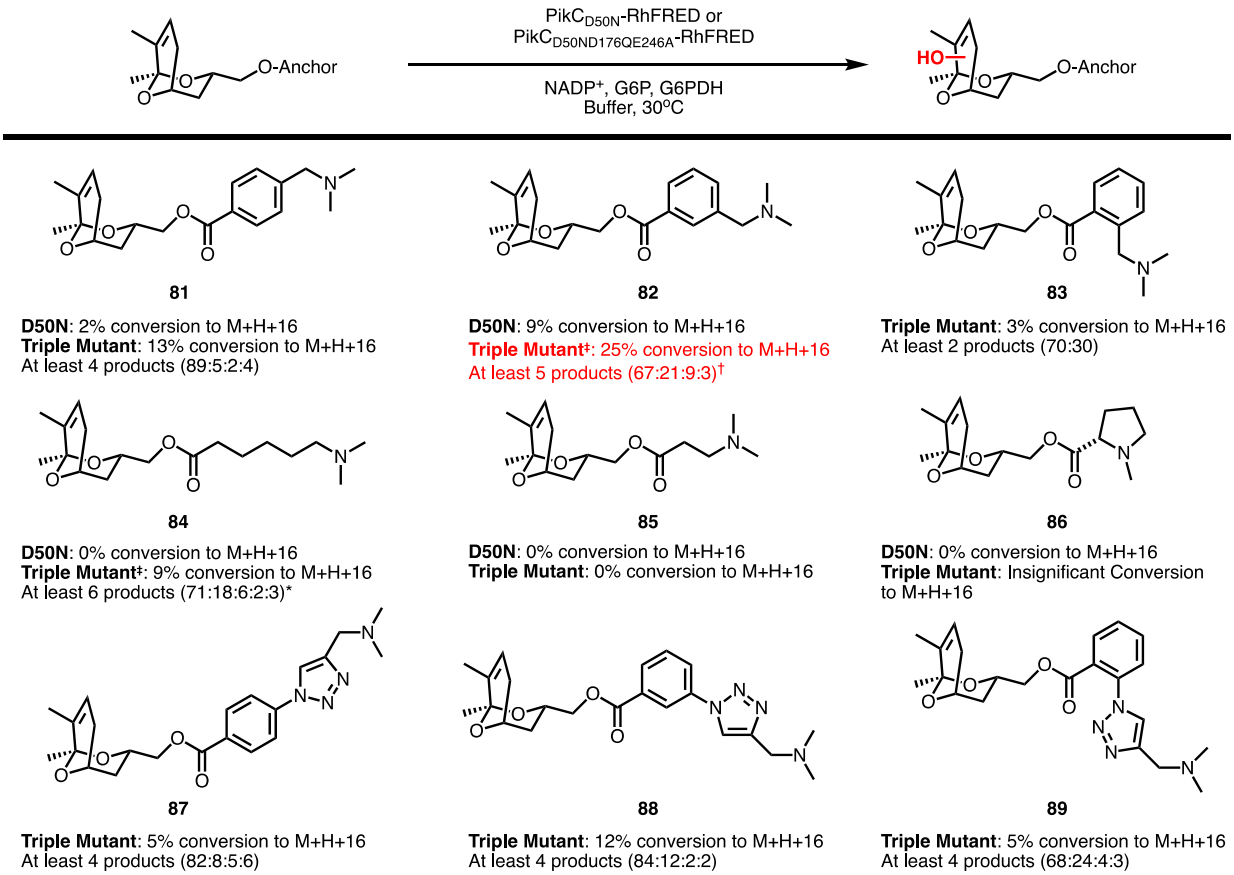
Table 3.2 – Enzymatic Oxidation of 1-Adamantanemethanol Derivatives by PikC$_{D50ND176QE246A}$-RhFRED

3.1.3.2 Enzymatic Oxidation of Tirandamycin Analogs with PikC

TirC analogs were synthesized as shown in Scheme 2.9 and attached to a collection of well-established PikC anchors, including benzylic amines, long and short chain linear anchors, nitrogen containing heterocycles, and 1,4-triazoles possessing

ortho-, meta-, and para-substituted benzene spacers (Figure 3.3). Compounds **81**, **82**, **84**, **85**, and **86** were first synthesized and reacted with two different PikC mutants which were utilized in previous studies[62,63,77] ($PikC_{D50N}$-RhFRED and $PikC_{D50ND176QE246A}$-RhFRED). Not surprisingly, increased conversions were observed for substrates **81**, **82**, and **84** with the catalytically superior PikC triple mutant. Enzymatic reactions with remaining substrates (**83**, **87**, **88**, and **89**) were therefore only run using $PikC_{D50ND176QE246A}$-RhFRED alongside a positive control reaction with (–)-menthol substrate **69**. Enzymatic conversions were determined from integration of the extracted ion chromatograms of LCMS traces, assuming equal ionization efficiencies of the starting material and products.

The lack of product formation observed for **85** and **86** can be rationalized through an observed lack of stability of particular tirandamycin analogs. **85** and **86** readily hydrolyzed in the enzyme buffer solution, as evidenced by lack of a significant M+H peak in the total ion chromatogram of these reaction mixtures. In addition, these compounds are not bench stable for extended periods of time (2 months or less). 1,4- and 1,5-triazole anchors are even less stable when attached to the tirandamycin bicycle (not shown); while the 1,5-triazole linked compound decomposes within a couple weeks of bench top storage, the 1,4-triazole anchor hydrolyzes upon standing within a few days.



‡ Reported as an average over two trials, † Ratio reported as a ratio of P1+P2:P3:P4:P5 due to overlap in P1 and P2 peaks,
* Ratio reported as a ratio of P1+P2:P3:P4:P5:P6 due to overlap in P1 and P2 peaks

Figure 3.3 – Reaction of TirC Analogs with PikC$_{D50N}$-RhFRED and PikC$_{D50ND176QE246A}$-RhFRED

Although enzymatic conversions to mono-oxidized product were low in comparison to former experiments with YC-17 analogs,[62] in certain cases, attachment of structurally rigid anchors such as *m*-benzylic amine **82** gave yields which were similar to those previously obtained for small molecule ring systems.[63] Meta-substituted arene spacers (**82** and **88**) out performed their para and ortho substituted counterparts (**81**, **83**, **87** and **89**). For example, m-aryl anchor **82** resulted in 25% conversion to M+H+16 as opposed to 1,4- and 1,2-substituted benzylic amines, which gave 13 and 3% conversion, respectively. LCMS traces typically showed conversion to at least one

major product, however, in most situations, a minimum of three minor mono-oxygenated products was also observed.

Fortunately, we found the selectivity and yield of meta-functionalized benzylic amine **82** to be sufficient to isolate and characterize two of the major reaction products (**90** and **91**) from a preparative scale reaction mixture (Figure 3.4A). While these two products were not effectively resolved in our initial LCMS chromatogram (both products co-eluted as part of first large orange peak in Figure 3.4B), we were happy to discover that both compounds were separable by reverse phase preparative HPLC. Oxidation occurred at the most electronically activated positions within the bicyclic ring system to give allylic hydroxylation at the C18 methyl (**90**) and C10 methylene (**91**). Notably, **91** was determined by 2D NOESY experiments to be the epimer of TirE analog **59** (Scheme 2.10), possessing the opposite C10 stereochemistry of what is obtained in the first step of the TamI-TamL oxidative cascade (Scheme 1.6). **90**, on the other hand, represents a new oxidation product that is structurally unrelated to any previously isolated biosynthetic intermediates. **90** is only the second reported example of primary C-H bond oxidation catalyzed by PikC, with (+)-α-terpineol derivative **70** being the first.[63]

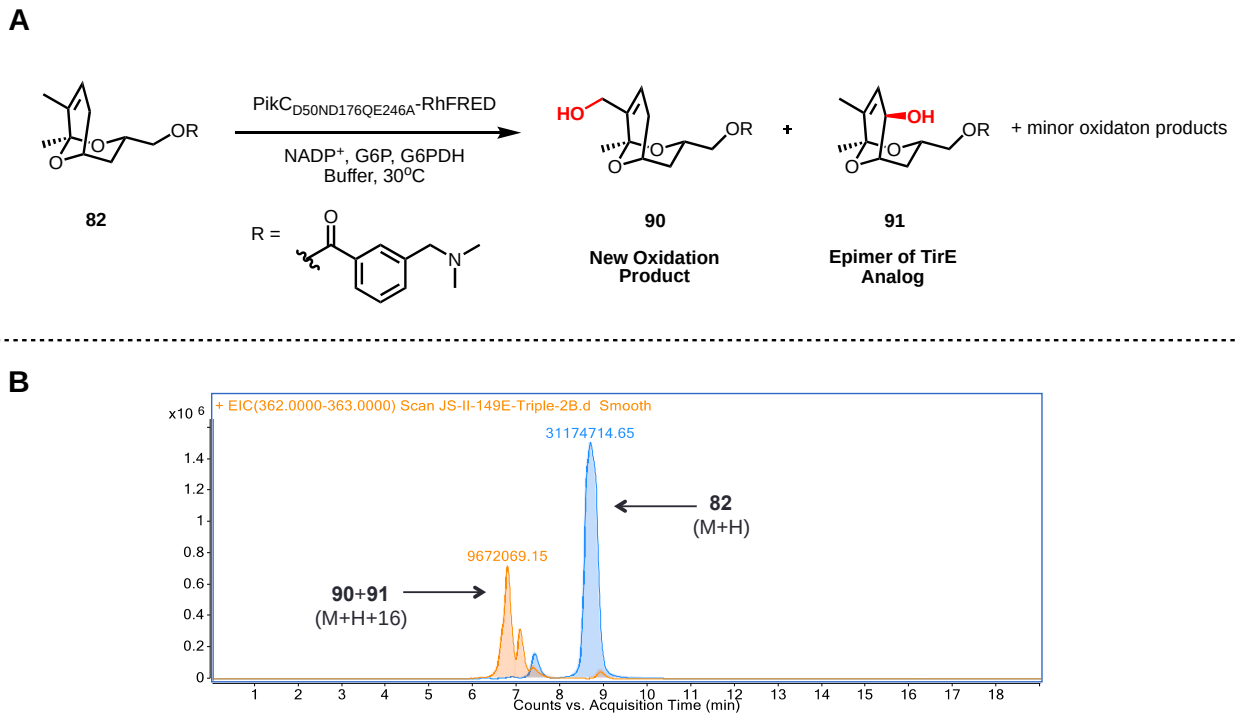


Figure 3.4 – Characterization of Major Oxidation Products from Reaction of a TirC Analog with PikC$_{D50ND176QE246A}$-RhFRED

We also sought to examine higher oxidation state tirandamycin analogs (Scheme 2.10) as substrates in anticipation that these structures, which mimic late-stage biosynthetic pathway intermediates, could potentially influence unique reactivity within PikC. In particular, we were especially interested in trying to replicate the TamI-catalyzed epoxidation of TirD with TirD analog **58** in order to expand the functional capabilities of the PikC enzyme.

To test the feasibility of our hypothesis, allylic ketone **58** was attached to 4-((dimethylamino)methyl)benzoic acid under the assumption that this anchor would provide similar conversions as previously shown with TirC analog **82** (Figure 3.3). 18% of this resulting material (**92**) was oxidized to two products with a desired mass of M+H+16 when subjected to PikC$_{D50ND176QE246A}$-RhFRED (Figure 3.5A). (Note that both

products co-eluted on the analytical scale LCMS trace as shown in Figure 3.5B.) This is different from other PikC catalyzed reactions with TirC analogs, which usually showed conversions to 4 or more different mono-oxidized products.

Unfortunately, less than a gram of each product was obtained from preparative scale reaction mixtures, making it impossible to fully characterize the isolated material by 2D NMR. Interestingly, the ^{1}H NMR of one product lacked the diagnostic singlet corresponding to the C11 vinyl proton around 6.2 ppm; however, neither proton NMR spectrum matched that of an epoxidized authentic standard. One alternative explanation could be formation of the exocyclic epoxide (as found in streptolydigin) through C18 hydroxylation followed by Michael addition to the neighboring olefin, although this has yet to be confirmed through future studies.

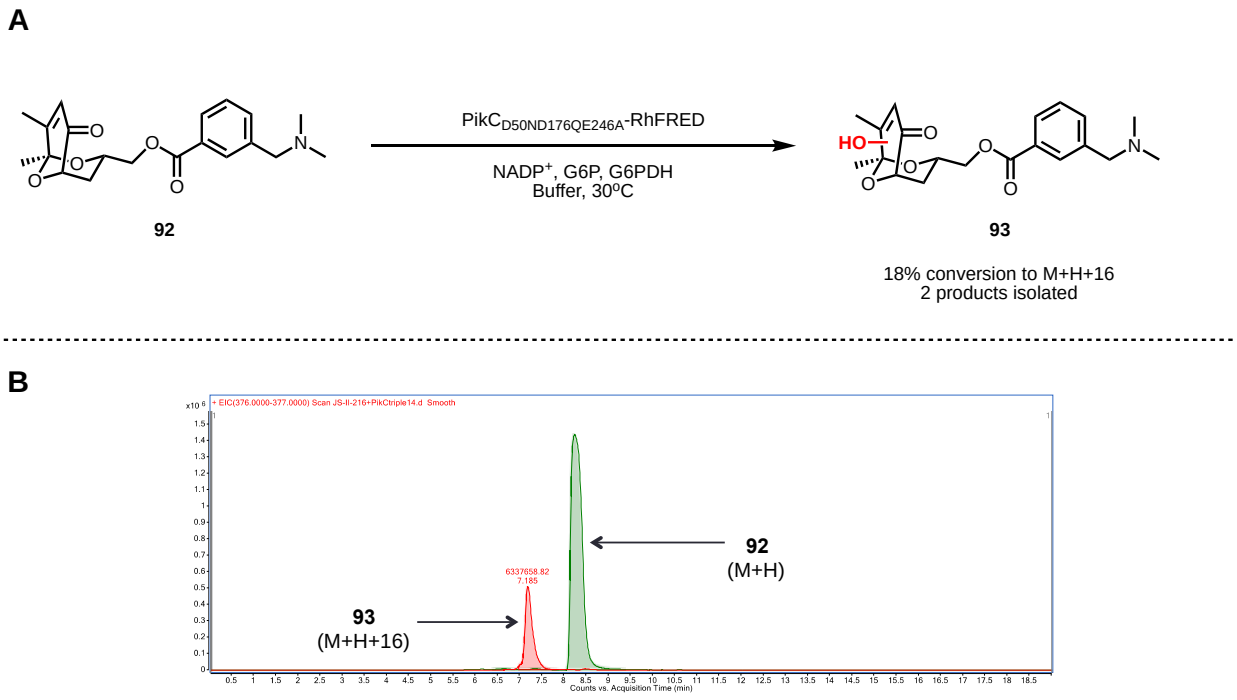


Figure 3.5 – Progress Towards Characterization of Major Oxidation Products from Reaction of a TirD Analog with PikC$_{D50ND176QE246A}$-RhFRED

3.2 Preliminary Work Towards the Use of TamI as a Novel, Multifunctional Biocatalyst

A secondary and perhaps easier approach to developing a general, mixed-function P450 biocatalyst would involve exploiting the versatile activity of TamI using substrate and protein engineering strategies similar to those previously utilized with PikC. Nonetheless, several potential limitations could hinder progress towards this goal, namely, unfamiliarity with the native binding mechanism and substrate promiscuity of TamI. In other words, if TamI does not anchor tirandamycin through several key-binding interactions but instead is highly specialized towards the catalysis of its endogenous substrate, this type of strategy will not be successful.

During the screening of tirandamycin analogs with PikC all substrates were also reacted with the TamI fusion protein, TamI-RhFRED (Figure 3.6). Not surprisingly, the majority of compounds did not show any conversion to oxidized product. However, meta-substituted benzylic amine **82** did show a minor amount of reactivity, indicating that unnatural substrates can be accommodated within the TamI active site.

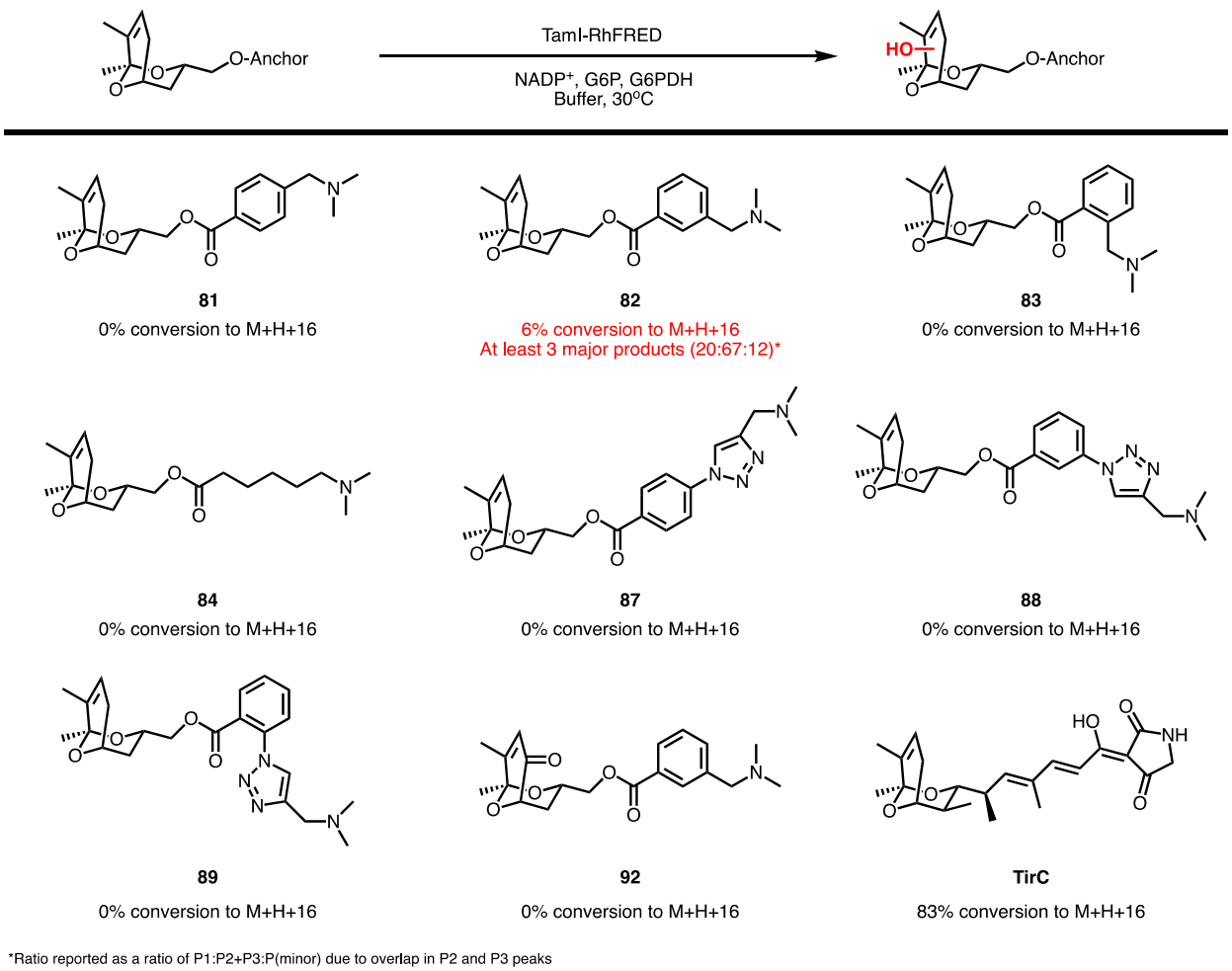
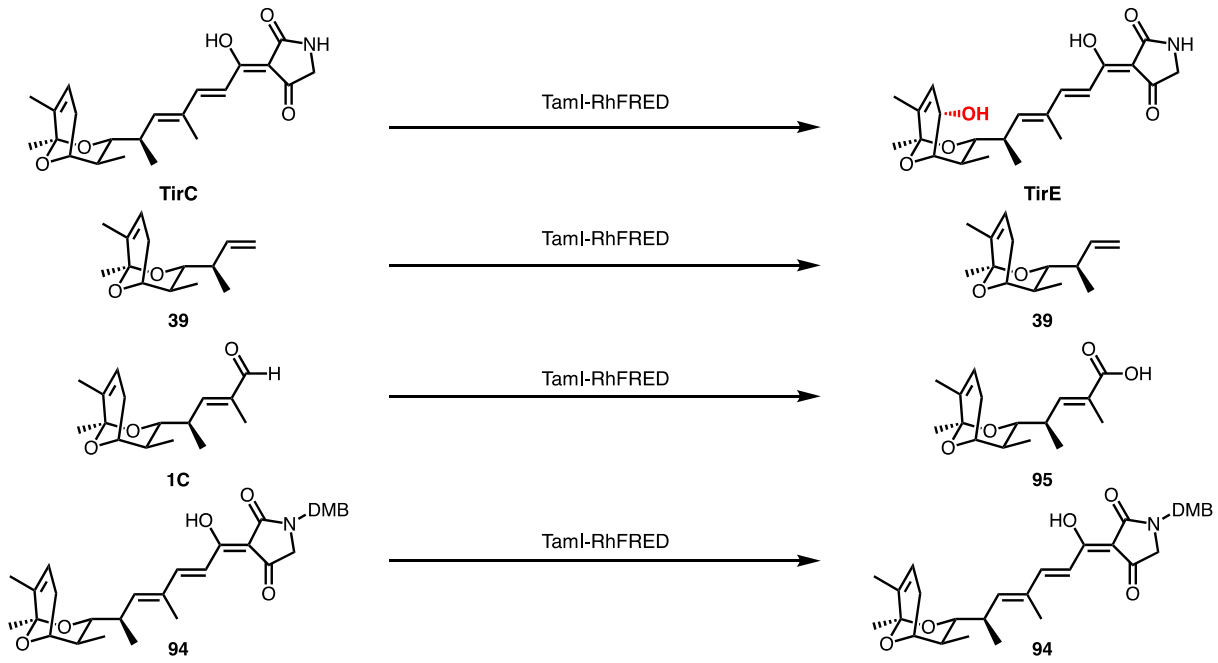


Figure 3.6 – Reaction of Tirandamycin Analogs with TamI-RhFRED

3.2.1 Insights into Substrate Binding and Anchoring Mechanism

Preliminary enzymatic experiments with late-stage intermediates from the total synthesis of TirC by Roush et al.[51] provided early evidence that the tetramic acid moiety may be important for catalysis with TamI (Scheme 3.2). These compounds, which were generously given to us by the Roush lab, were tested as substrates for TamI-RhFRED in order to probe the structural requirements necessary for expanding the substrate scope of TamI. Interestingly, only the TirC control was oxidized *in vitro*, while tirandamycin derivatives that lacked the tetramic moiety (**39** and **1C)** showed no

reactivity. This potentially suggests that, similar to desosamine,[72] the tetramic acid of tirandamycin serves as an anchor which is responsible for orienting and binding substrates in TamI. Furthermore, the lack of reactivity of DMB-protected tetramic acid **94** could indicate that the N-H bond of the 2,4-pyrrolidinedione ring system forms a necessary electrostatic interaction with catalytic residues in the binding pocket of TamI; however, it is more likely that the benzyl protecting group is too sterically encumbering to be accepted into the enzyme active site.



Scheme 3.2 – Reaction of Late-Stage Roush Intermediates with TamI-RhFRED

Obtaining crystallographic information on the protein structure and binding mechanism of TamI has long eluded our groups' research efforts. Original attempts at crystallizing TamI resulted in a crystallographic dimer structure via an engineered N-terminal overhang coding for a His-tag[40] (Figure 3.7A). Relocation of the His-tag to the C-terminus of the protein had no positive effect on crystallization.

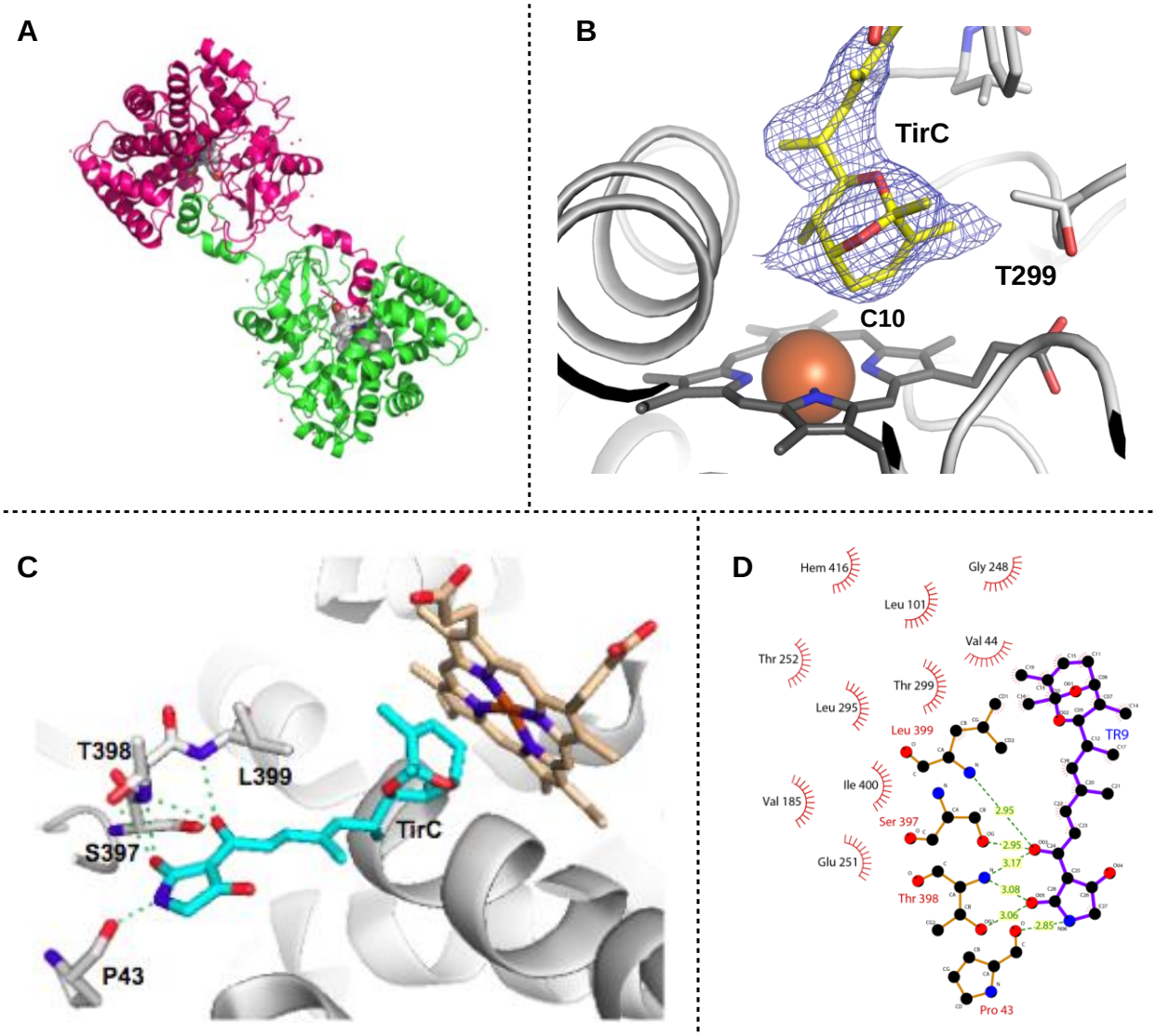


Figure 3.7 – TamI Crystal Structure **A:** TamI Crystallographic Dimer (*Unpublished work by Dr. Larissa Podust*) **B-D:** TirC Bound Structure of TamI (*Unpublished work by Dr. Sean Newmister*)

Members of our research team hypothesized that cleaving the N-terminal His-tag may free access to the active site. Consequently, TamI was engineered with a cleavable N terminus leading to successful co-crystallization with TirC. The resolved structure reveals several valuable insights into the origin of binding and selectivity in this

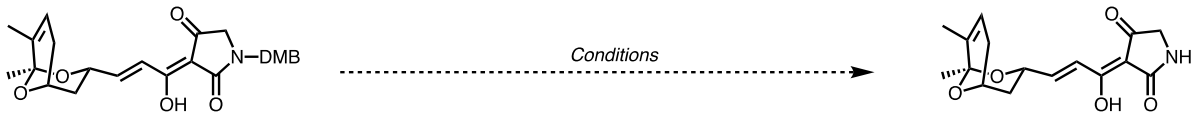
multifunctional P450. As expected, the orientation of the tirandamycin bicycle matches its observed reactivity, with the C10 allylic methylene of TirC situated 4.1 Å away from the iron heme center (Figure 3.7B). Furthermore, non-specific stabilizing interactions allow for flexibility in size and orientation of the bicyclic ketal. This aligns well with the proposed biosynthetic pathway of tirandamycin, which requires the repositioning of the bicycle in order to facilitate sequential oxidation.

The tetramic acid moiety is much more tightly packed in the TamI active site and seems to be held in place through potential hydrogen bonding interactions with several amino acid residues, including Leu-399, Ser-397, Thr-398, and Pro-43 (Figure 3.7C/D). While the backbone of Leu-399 and alcohol side chain of Ser-397 appear to form a tight contact with the C1 enol of tirandamycin, the backbone of Thr-398 is positioned to engage in similar interactions with the C1 enol and the C2' carbonyl of the tetramic acid. The tetramate nitrogen was also identified to potentially aid in substrate binding through association with the backbone of Pro-43. These crystallographic insights, in combination with preliminary computational docking efforts with other tirandamycin congeners, further suggest that an anchoring mechanism may be operational.

3.2.2 Work Towards the Synthesis and Design of TamI Anchors

As mentioned previously, tirandamycin analogs attached to PikC anchors showed minimal to no reactivity with TamI-RhFRED (Figure 3.6). Motivated by recent findings from the TamI crystal structure, we were interested in seeing if incorporation of a tetramic acid moiety would improve enzymatic conversions and validate our hypothesis in support of an anchoring mechanism. TirC analog **96** was synthesized through DMP oxidation of bicycle **57**, followed by Horner-Wadsworth Emmons

Olefination with phosphonate tetramic acid **3** (Scheme 2.1A). Deprotection of the DMB group, however, proved challenging (Table 3.3). A variety of different reaction conditions were screened following previously reported procedures.[44,51,52] In all cases, decomposition of the starting material was observed by ^{1}H NMR with no evidence of conversion to deprotected product. Decomposition of the unanchored bicycle was also observed after stirring in TFA/DCM at 0^{o}C for 5 minutes. In the future, more labile protecting groups could be used to remove the amide substituent under milder conditions.



96 **97**

Entry	Conditions	Time	Concentration*
1	TFA, Anisole, RT	1 hr. 10 min.	0.03 M
2	TFA (neat), RT	15 min.	N/A
3	TFA, DCM, RT	30 min.	0.02 M
4	TFA, DCM, RT	45 min.	0.026 M
5	TFA, DCM, RT	5 min.	0.013 M
6	TFA, DCM, 0 ^{o}C	5 min.	0.012 M
7	TFA‡, Anisole, 0 ^{o}C	5 min.	0.009 M

*Indicates concentration of substrate in reaction solvent
‡TFA was distilled before use

Table 3.3 – Attempts at Deprotection of DMB Group

New insights into potential binding interactions in TamI have inspired the design of more compatible synthetic anchors, which incorporate key structural features (e.g. primary amines, amides, and 1,3-dicarbonyls) predicted to be important for substrate binding (Figure 3.8). Anchors will be synthesized either through simple modifications of PikC anchors (**98**, **99**) or derivatization of other readily accessible core scaffolds, such as cyclic ureas.

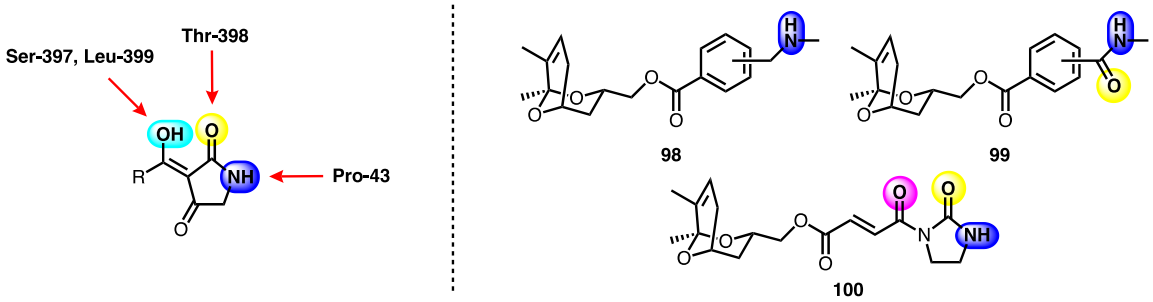


Figure 3.8 – Design of Synthetic TamI Anchors

3.3 Conclusions and Future Directions

Overall, the work described in this chapter highlights the development of cytochrome P450 enzymes as site-selective biocatalysts for multi-functional C-H oxidation. Tirandamycin analogs were effectively oxidized by PikC to afford novel oxidation scaffolds which are not readily accessible through natural product pathways, thereby illustrating another unnatural substrate class for this versatile P450. Furthermore, TamI has shown early promise to be used as a complementary P450 for multifunctional C-H oxidation. While the complex factors governing mixed-function behavior in P450s are still not yet fully understood, future work involving a combination of computationally guided substrate and protein engineering efforts will lead to increased knowledge in this area. This will include both rational mutagenesis and synthetic manipulation of tirandamycin and its analogs to elucidate key structural features important for binding and activity, making further progress towards the use of enzymes as a tool for the diversification of a broad range of target molecules.

3.4 Collaborator Acknowledgements

Dr. Alison Narayan and Dr. Solymar Negretti Emmanuelli are acknowledged for their substantial contributions towards the development of the PikC biocatalytic system. Dr. Alison Narayan performed the enzymatic reactions in Table 3.2 and Scheme 3.2. Dr. Matthew DeMars performed the enzymatic reactions in Figures 3.3 and 3.6 and helped with isolation of compounds **90** and **91**. Dr. Sean Newmister obtained the TamI crystal structure shown in Figure 3.7B-D.

CHAPTER IV

Investigating the Benefits of a R1/PUI Laboratory Exchange Program Related to Graduate and Undergraduate Student Learning and Professional Development

4.1 Introduction

While the synthetic studies presented in chapters 1-3 of this thesis have played a major role in my graduate career, I have also had the opportunity to design and implement an educational research project which has focused on learning in the laboratory environment. Specifically, this project involves a two-part student exchange between the Montgomery* laboratory and an undergraduate lab at Hope College in Holland, MI that was established in order to help provide exchange program participants with valuable professional development experiences such as working in or managing a research laboratory.

The idea for a laboratory exchange program was originally proposed as an initiative to help supplement the career development training offered to graduate students through the University of Michigan chemistry department. A long-standing challenge in the education of future faculty members in the sciences is that professional preparation often focuses heavily on students' training as scientists at the expense of their training as educators. As Coppola[78] argues:

*Unless otherwise noted, permission was given to disclose participants' identity.

> [T]he extent of professional readiness required for discovery research…should be, if not must be, broadened to include the full array of responsibilities and obligations for a member of a faculty on the first day of one's independent career…If we trained physicians the way we train professors, we would never let them see a patient or use a stethoscope before they stepped into their first practice.

Arguably, major research institutions such as the University of Michigan are better positioned to provide support for students looking to pursue careers in academia at an R1 research university[79] since faculty have direct personal experience establishing themselves in this type of setting. However, in the field of chemistry for example, only 190 out of 5000 colleges and universities grant doctorate degrees, with almost 50% of Ph.D. students enrolled at only 30 of these schools.[79,80] It is therefore unlikely that prospective chemistry professors will end up teaching in an environment similar to their graduate institution.

Providing assistance to graduate students looking to teach at primarily undergraduate institutions (PUIs) is not straightforward either. Although departmental seminars and workshops sponsored by internal professional development infrastructure[78] can help bridge the gap, this does not serve as a sufficient replacement for hands-on, in-person experience working in the unique atmosphere of a private, liberal arts institution. Furthermore, even students who may be familiar with the PUI culture from their undergraduate studies likely were not focused on learning how to serve as a faculty member during their time there. Creating an opportunity for graduate students to work alongside teachers and students at a PUI for an extended period of time during their Ph.D. candidacy would therefore be beneficial to students, especially before they commit to this particular career path.

With respect to undergraduates, there are a wide variety of exchange program opportunities available to undergraduate students looking for discipline-specific research experiences in science, technology, engineering and math (STEM). The National Science Foundation's (NSF) Research Experiences for Undergraduates (REU) program, for example, began in 1987 in an effort to encourage undergraduate engagement in authentic research practice and continuing education into graduate school.[81] Typically, REU students are given a stipend to travel to a host institution and work on an NSF-funded research project under the guidance of faculty and graduate student mentors. Similar study abroad opportunities are offered through the RISE (Research Internships in Science and Engineering) Program, Trans-Atlantic Science Student Exchange Program (TASSEP), and the Fulbright Program, among others.[82]

The benefits of undergraduate research are well studied in the literature, having a strong influence on students' future career decisions, scientific understanding, and research and communication skills.[81,83–86] However, one-time, 10-week exchange experiences, such as the ones supported through the REU program, make it difficult for students to form long-lasting, sustainable interactions with their assigned mentors, given that these programs are relatively brief and have no mechanism in place for continued communication beyond the 10-week period. Work by Thiry and Laursen[87,88] suggests that this is an important factor to consider, showing a significant correlation between the amount of time spent with research mentors (typically identified as either graduate students, postdoctoral researchers, or the principle investigator of the laboratory) and students' overall satisfaction and intellectual gains. Furthermore, Sadler et al.[89] noticed

a similar correlation between the length of research experiences and participant learning outcomes.

Prior to starting this work, the Montgomery lab had been fortunate to establish a relationship with Professor Jeff Johnson's laboratory at Hope College through mutual research interests, relative geographic proximity, and an overlap in student workers (i.e. students who graduated from the Johnson lab and then worked as graduate students in the Montgomery lab). Hope College is a Christian, liberal arts college located in Holland, MI with a student body of approximately 3,000 undergraduates and an internationally recognized chemistry department.[90] In the ten years that Dr. Johnson has been a professor at Hope College he has mentored over fifty undergraduate and visiting high school students, many of whom are co-authors on high-impact publications stemming from their research accomplishments in his lab.[91,92] His professional accomplishments in teaching and research have been recognized through his receipt of numerous awards, including the prestigious Henry Dreyfus Teacher-Scholar Award in 2015.

The objective for initiating an R1/PUI student exchange was to create a program that would meet the unique needs of both graduate and undergraduate students while simultaneously reinforcing continual interaction between our two research groups. We envisioned that this exchange program would provide invaluable career development training for graduate students by allowing them to learn directly from an undergraduate instructor about how to manage the different responsibilities associated with a career at a PUI, such as running an undergraduate research laboratory. Similarly, the undergraduate student participant would hopefully gain a more informed perspective on

graduate school at an R1 institution and develop important research and critical thinking skills.

We purposefully chose to organize this exchange sequentially over the course of two summers, with a graduate student visiting Hope College for four weeks the first summer and then mentoring a member of the Johnson lab at the University of Michigan for 10 weeks the following year. By extending the exchange program over a two-year period and providing the graduate student the chance to observe effective laboratory teaching practices prior to mentoring the undergraduate in the R1 research setting, we hoped to improve both the quality and quantity of student-mentor interactions. Furthermore, we believed that giving the graduate student first-hand perspective on the undergraduate student's background knowledge and experiences prior to working with him or her in the Montgomery lab would contribute to an overall more effective learning experience for both parties involved.

In the summer of 2015 I was fortunate enough to be given the opportunity to participate in the first iteration of the Montgomery/Johnson lab exchange and work alongside Stanna Dorn, a Hope College undergraduate going into her junior year as a chemistry major at the time. The remainder of this chapter will focus on describing what we learned from this experience, which was analyzed as part of a case study in order to investigate whether or not our design of this exchange program had the impact we intended. Specifically, the research questions we were interested in answering were:

What can a graduate student and a PUI undergraduate learn from visiting each other's laboratories that is beneficial to their professional development? How did this learning occur?

4.2 Background

Laboratory learning is well characterized by situated learning theory, a perspective first introduced by Lave and Wenger[93,94] which describes learning as being situated in specific communities of practice, and mediated by experiences, practices, norms, tools, and relationships with others. In this sense, learning is situated within the same context in which it is applied, as is embodied by apprentices in various conventional trades (e.g. tailors, midwives). In other words, instead of potentially passive learning from a textbook or lecture, learning occurs through ongoing engagement with a group of people working and learning together towards a common interest. "Newcomers" to this community learn through "legitimate peripheral participation," completing tasks that are necessary, although not central, to the overall functioning of the community. For example, Lave[95] describes how apprentice Liberian tailors must start by ironing garments before learning how to sew. Furthermore, gradual socialization into a community of practice is not only associated with mastery of a particular skill-set, but a change in identity as a master practitioner of the trade.

Mentoring is a key component of any apprenticeship. Direction and support from a mentor help an apprentice advance their learning beyond their independent capabilities, a concept well explained by Vygotsky's zone of proximal development (ZPD).[96] According to Vygotsky, the ZPD is the difference between what a learner can do independently and what a learner can do with the help of a more experienced other. This helps the learner work towards greater autonomy and proficiency in a certain area, until he or she no longer needs the support provided by others but can instead serve as an advisor to newer members of the community.

Scaffolding can be an important set of supports to help learners make progress toward solving a problem or completing a task which would otherwise be outside the limits of their current potential.[97] An early example of scaffolding as described by Jerome Bruner[98] includes the use of picture book reading by parents to help young children acquire meaning when learning how to speak. In general, scaffolding occurs by 1) initially reducing the difficulty of the task at hand, 2) focusing the learner's attention on relevant details, and 3) modeling desired behaviors towards completion of a task. The scaffold is then gradually removed as the learner becomes more knowledgeable.

Academic research laboratories are an example site for modern-day apprenticeships. The training of young research scientists is not unlike that of traditional apprentices; just as Liberian tailors[99] must learn through observation of their elders and through the completion of tasks that are legitimate and peripheral to the practice of tailoring, new students in a research lab must also learn basic skills by working closely under the supervision of a more experienced mentor. This typically involves novice student researchers repeating known or simple experimental procedures before starting to work independently on their own original research projects.

Several studies have analyzed how learning occurs through secondary and post-secondary research apprenticeships.[88,89,100–105] However, to the best of our knowledge, similar research has not been conducted on laboratory exchange programs in which members of two or more participating research groups work in each other's laboratories for an extended period of time. As noted by Lemke,[106] time is an important factor to pay

attention to when studying learning, especially when considering issues of identity development, which take place over longer timescales. By focusing on participants' personal growth over the course of two separate summers, we argue that more long-term learning trajectories will become increasingly visible. Furthermore, as mentioned earlier, the collaborative nature of an exchange program creates the opportunity for extended interaction with mentors, which may confer additional learning benefits.[87,88] Therefore, studying exchange programs as research apprenticeships, and thus, sites of science-related learning and identity formation, is an important contribution to the literature. We hypothesize that by immersing oneself in a different, albeit related, community of practice, exchange program participants have access to learning opportunities that they would not ordinarily have.

4.3 Methods and Analysis

Our research was conducted as a two-part case study to coincide with both stages of implementation of the exchange program, i.e., my visitation to Hope College in June 2015 and Stanna's visit to the University of Michigan the following summer. Case studies provide an in-depth investigation of a bounded system (e.g. people, event activity, program) that is delineated in terms of time and space in order to provide a finite limit to data collection and analysis.[107,108] In this particular instance, the focus of our case study was bounded by the 14-week time period of the Montgomery/Johnson lab exchange program and concentrated on exploring the learning benefits of only its main participants (myself and Stanna). Despite the importance of defining boundaries around the object of study, often times in case studies the boundaries between phenomenon and context are not obvious.[109] This drastically opposes most

experimental studies, in which the setting is typically controlled for in order to study only a handful of variables. Case studies, on the other hand, frequently involve a large number of interconnected variables which are evaluated through the triangulation of multiple sources of evidence. In this study, extensive collection and analysis of interview data, field notes and reflections allowed for a more thorough understanding of specific details and learning processes that would not otherwise be captured through quantitative methods.

Since the sample size of a case study is so small (n =1), our findings were not intended to give generalizable results that would necessarily apply in an investigation of other exchange programs, but instead were intended to contribute to the literature on how learning occurs in these types of settings. We anticipated that these results would not only be useful for re-design of future iterations of the Montgomery/Johnson exchange, but also hoped that positive outlooks from this study would inspire similar initiatives between other laboratories and provide support for future funding of these opportunities.

During my time at Hope College I conducted a series of four different semi-structured interviews with select members of the Johnson laboratory, including Dr. Johnson himself. The semi-structured[110,111] nature of the interviews ensured conversations stayed focused and consistent, while still providing flexibility for follow-up questions and elaboration of student responses where necessary. Interview questions were pre-written before the start of the exchange and centered on topics such as the group dynamic, Professor Johnson's mentoring style, and students' experiences

working and learning in the laboratory. All of the interview questions used in this study are included in chapter 5 of this thesis (supporting information).

Students who were interviewed were selected from a variety of backgrounds and expertise, taking into consideration differences in grade level, gender, and years of research experience. Pseudonyms are used to protect the identity of these students, with the exception of Stanna, who gave us permission to use her real name in order to acknowledge her significant contributions towards both this project and other aspects of this dissertation research, which were previously disclosed.

As summarized in table 4.1, the four students I interviewed were Stanna, Karen, Lucy, and Ben. Stanna and Karen agreed to be interviewed together as part of a small focus group since they were close friends and felt comfortable speaking candidly in front of one another. At the time of the interview, Lucy was going into her junior year as a chemistry major at Hope College and was the most senior member of the Johnson lab due to her participation in the Research Experience Across Cultures at Hope (REACH) program, which allowed her to work in Professor Johnson's lab for two summers as a high school student. Ben, Stanna and Karen were all in their first year working in the Johnson lab, however Stanna had experience working in a different laboratory the previous summer. Both Stanna and Karen were soon-to-be juniors whereas Ben was going to be a senior at the time that this research was conducted.

Participant	Gender	Ethnicity/Citizenship	Year in College (Starting Fall '15)	Previous Research Experience
Stanna	Female	Caucasian / US (Michigan Resident)	Junior	1st summer working in Johnson lab, 2nd summer conducting research at Hope
Lucy	Female	Caucasian / US (Michigan Resident)	Junior	4th summer working in Johnson lab
Ben	Male	Asian / Chinese Citizen	Senior	First research experience
Karen	Female	Caucasian / US (Indiana Resident)	Junior	First research experience

Table 4.1 – Johnson Lab Interview Participants

While interview data served as our primary source of data from this portion of our case study, field notes[111–113] were also recorded to document the 4-week visitation. These consisted of observations of lab members' interactions, summaries of my daily experiences and conversations with others, and personal learning reflections in response to these occurrences. These insights were also incorporated into our analysis of the exchange program.

Interviews were used to more closely reveal Stanna's perception of the exchange program throughout her time in the Montgomery lab. Interviews were conducted at the beginning, middle, and end of the visitation at one, five, and ten weeks, respectively, and focused on topics such as expectations of the exchange program, difficulties and struggles that occurred along the way, and perceived personal and professional learning outcomes. A brief follow-up interview was also conducted in order to gauge the impact of the experience two months following Stanna's return to Hope.

All interviews used throughout the study were recorded and later transcribed. Emergent themes were then identified across the entire data corpus, including interviews from both segments of the exchange program and accompanying field notes. Codes were limited to themes that were prevalent across the entire data set, provided

bearing on the research questions, and were well aligned with outcomes which were previously identified in the research apprenticeship literature.[88,89,100–105]

4.4 Results and Discussion

4.4.1 Hope College Visitation

Early in June 2015 I came to the Johnson laboratory interested in learning what factors make Dr. Johnson's research program so "successful," as evidenced by: 1) the large number of publications Professor Johnson has co-authored in the short amount of time since starting his independent career, and 2) the far-reaching personal impact he has on his students, seen through the fond memories they share long after graduating. I hoped that by studying first hand how the Johnson lab functions, this experience would provide me with valuable insights that would someday be useful when starting my own research laboratory.

Most of the lab's accomplishments can be linked in some way to its purposeful structuring into a community of practice.[93,94] Despite being comprised entirely of undergraduate and visiting high school students, the Johnson lab functions much the same as a typical academic laboratory at an R1 institution, with more expert students serving as mentors for new lab members. This structure allows for the lab to overcome critical limitations that often plague PUI laboratories, such as lack of dedicated research time and student experience. By intentionally assigning certain students to leadership positions, Professor Johnson is able to manage a larger number of students, while staying focused on matters that are more important for the overall organization and success of the laboratory. As he describes:

I put a lot of emphasis on when students are returning that they take leadership

> roles, whether official or unofficial, to kind of mentor new students, and I still keep an eye on students and what they're doing, of course, cause you don't want people teaching the wrong thing, but to try to get that sort of system going, and have a "critical mass" of returning students each year, then it definitely takes a lot of the day-to-day, "*Ok, now where do I find the test tubes?*" or "*Where do I find the solvent?*" and pushes that off on other students who can handle that sort of thing.

Lab members support each other in many ways other than just helping find test tubes and solvent, however. Karen, for example, commented on how she felt the relatively large size of the Johnson lab leads to the exchange of multiple, diverse viewpoints which would not otherwise be visible if students worked independently of one another. This in many ways is reminiscent of Vygotsky's zone of proximal development, in which learners' move beyond their normal capabilities through the assistance of others:[96]

> I think that because he has so many of us, it means that you get more perspectives than you would otherwise. So I know that sometimes we'll be sitting and someone will suggest something that I never would have thought of, and then someone else will suggest something else that no one else in the group ever would have thought of, and it's just we all have different experiences. And to a degree, a lot of our chemistry experience is similar but everyone thinks in different ways. So by making us all part of it, he's getting all those different perspectives. And it also means that we can just push through [research]…. So I think some of it is just having that many people speeds up the [research] process.

Others outside of the laboratory seemed to recognize the benefits of this shared sense of community as well. Stanna, for example, recalled how observing Johnson lab members' interactions while working in a different lab her first summer at Hope made her want to be included:

> Last summer I got to watch the whole Johnson group dynamic from the outside and I saw how tight-knit everybody was and I was like, *"Wow, I would really love to be a part of a community and I feel like I would benefit from that kind of community."*

The research apprenticeship literature has identified a number of personal and professional gains associated with newcomers' socialization into a research community, including increased understanding about the nature of science (NOS), intellectual growth, and impact on participants' career aspirations.[89] Similarly, we discovered several specific elements from our data that are important components of the Johnson lab's community of practice: the development of trust among lab members, and an increase in students' confidence, independence, and enjoyment in engaging in authentic scientific research (Figure 4.1).

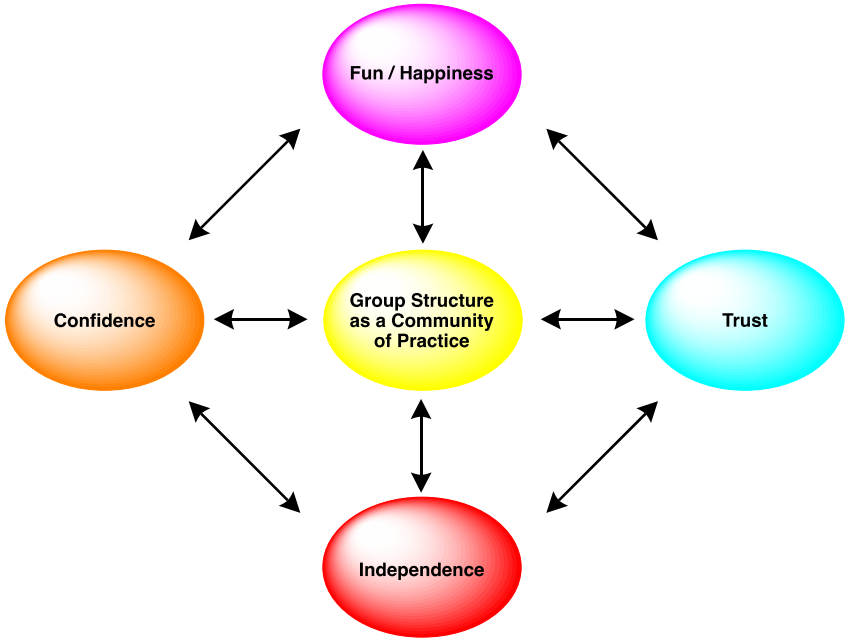


Figure 4.1 – Elements Linked to Johnson Lab Group Structure as a Community of Practice

As shown in Figure 4.1, we not only noticed a relationship between each of these elements and group structure, but also an interdependency of elements on one another. For example, as students gained more autonomy in designing experiments and

troubleshooting problems, they also became more confident in their abilities, something that Stanna, Lucy and Ben all said they struggled with in the beginning. Establishing a foundation of trust in the lab was also important in helping students feel more comfortable to take intellectual risks. The independence that Dr. Johnson gives his students is taken as a sign of his trust in them, which in turn helps them to grow as scientists. As Ben stated:

> The second time I would be doing [a procedure] all by myself because I feel like he trusts us that giving us instructions and also combining our lab mates' knowledge, we can reproduce results the second time from what he told us and what we observe[d]…I just feel like we are so self-sufficient and able to do so many things because [Dr. Johnson] trusts us with our abilities and in return we don't want to squander his trust.

Almost all students mentioned the importance of finding enjoyment and happiness in their work, particularly in their interactions with others. This was also consistently apparent from observations of the students, who took time during lunch to go outside and play lawn games, maintained an on-going game of chemistry Scrabble, and organized an entire dress up week where lab members came to work in themed costumes. The enjoyment of these social practices is a cherished tradition shared by members of the Johnson lab. While these activities may seem somewhat counter-productive and distracting, these customs create a more comfortable and welcoming atmosphere for new students and lead to the development of trusting, collegial relationships among lab members. Furthermore, Dr. Johnson also attributes this laidback behavior as a contributing factor in his students' success. As he mentioned in his interview with me, "These kids are far more productive because they're goofing off all the time than some other labs in the area where they're kind of under the thumb."

Table 4.2 provides additional empirical support for the identification of these four major elements (confidence, independence, trust, and fun/happiness) by interview participants. Although the specific learning gains of these individuals were not explicitly studied in this context, these attributes are unequivocally linked to several important aspects of learning, as described in the National Research Council's (NRC's) *How People Learn*.[114] For example, students reported increases in confidence through receiving peer feedback, and developed a sense of comfort in asking questions and taking intellectual risks. These elements are well supported through community-based teaching practices,[94] which cannot only lead to an increase in cognitive development, but can help challenge students' own misconceptions about how they learn.[115]

Element	Participant	Example of Instance Where This Element Was Described
Confidence	Lucy	"But everyone was so good [to me] and I learned how to do things, got some good feedback, and that really just helped me figure out that yeah I can do this and I like this."
Independence	Ben	"I guess it gives me a sense of independence whenever I [do] something correctly without his mentor[ship]…it's self-fulfilling."
Trust	Stanna	"I feel like amongst the whole lab there's a feeling of trust. You can go ask people [questions] and it's ok to fail. That's research. You can't be afraid."
Fun / Happiness	Lucy	"There's a good precedent in this lab, I think, of doing things together and having a lot of fun and so I think that makes the group dynamics really good. Sometime back when I started I wondered whether I actually liked the chemistry or just hanging out with the group more and I've decided I just really like both."

Table 4.2 – Descriptions of Confidence, Independence, Trust and Fun / Happiness from Student Interviews

In order to better understand my own personal impact from the exchange, we turned to analysis of field notes and personal reflections which were maintained over the course of the 4-week visitation experience. These data focused on many of the same emergent themes which were identified through participant interviews, including

group size/structure, independence, and fun/happiness. Some of these observations focused on similarities and differences I noticed between the Johnson laboratory and my own prior research experiences. In many ways the Johnson lab reminded me more of the Montgomery lab than the PUI lab I worked in as an undergraduate, which consisted of only 2 or 3 students and was much more closely supervised. As mentioned earlier, the Johnson lab seemed to model an R1 research group through its size, organization, and established practices. For instance, students functioned relatively independent of Dr. Johnson, as shown by their sustained productivity in his absence. Instead of relying on Dr. Johnson for assistance or approval, students' arrangement into different project-focused sub-groups provided support for members to work together towards achieving research goals, which were then presented and discussed with the entire lab during weekly group meetings.

More importantly, I learned that students appreciated this group structure and preferred being given the opportunity to work independently and learn from their own mistakes. This immediately impacted how I interacted with others in the Johnson laboratory, especially a high school student who I was in charge of mentoring at the time. Instead of intently watching over the student or repeatedly telling him what to do (as I would have previously done), I gave him the freedom to learn through guided discovery,[116] still providing assistance and teaching him the appropriate research and safety skills when necessary. This carried over into how I mentored Stanna the following summer in the Montgomery laboratory, allowing her to quickly grow into a more independent researcher (see section 4.4.2 below).

The widespread benefits of fostering enjoyment within the laboratory community also became much more apparent to me after working in the Johnson group. Beyond promoting happiness, I realized that encouraging students to have fun leads to an increase in trust among lab members and inspires the confidence to take risks. As I wrote on one of my last days at Hope, “Doing chemistry research doesn’t have to be a stressful, intense experience but instead should be FUN, especially when teaching and learning at this level.” This is a concept I have since tried to incorporate in my own teaching practices and hope to one-day apply as a foundation for my future research group.

My personal learning through this experience was influenced by a variety of factors, including my own engagement in the Johnson lab community of practice.[93,94] Just as Johnson lab members were informed by the customs and routines shared by others in the community, I too learned by working in the laboratory as a part of their group. This enabled me to acquire information that I ordinarily wouldn’t have through outside conversations with lab members; instead, developing a relationship with students in the lab allowed them to trust me with valuable information and observe intimate details of their day-to-day lives. Furthermore, recording my thoughts and experiences through periodic reflection employed meta-cognitive learning strategies[114] which led me to actively consider my thinking and better recall these ideas at a later date.

4.4.2 University of Michigan Visitation

Stanna’s 10-week visit to the Montgomery lab can similarly be characterized as a research apprenticeship. Despite her previous research experience, Stanna’s relative

unfamiliarity with organic synthesis made it important for her to first repeat known chemistry before transitioning to her own independent project. This involved synthesizing starting material that would later be used to make more significant research advancements in the laboratory, therefore embodying Lave's principle of legitimate peripheral participation.[93,94] Furthermore, this exercise also served as a scaffold[97] to help Stanna learn new techniques and become more comfortable with procedures in which she had minimal training. One week after her arrival, she described her expectations for the exchange program, which mirrored apprenticeship theories of learning:

> I'm just really excited to see and pick up on [group members'] lab technique and it's really fun just to kind of watch you guys… [It's been nice] picking up on how you guys set up reactions and decide when to do things. I also really enjoy hearing you guys talk to each other about different things.

As most commonly examined in the literature,[89] undergraduate research experiences typically result in significant professional development gains, including growth as a researcher and increased preparation for a career in the sciences. Stanna noted similar learning outcomes, such as the development of fundamental research skills and laboratory techniques as detailed in the first half of Table 4.3. For example, Stanna frequently commented on feeling more comfortable with purification methods such as column chromatography and learning to use thin layer chromatography (TLC) as a means to track the progress of a chemical reaction. Many of these new research procedures were documented in a "daily diary" which Stanna reported helped to support her learning by allowing her to better remember information. In other words, by writing notes and drawing figures of new laboratory techniques Stanna was able to make her learning experiences more permanent and useful to herself in the future. She also

mentioned that participating in group-based problem solving sessions and focusing on more exploratory research aims helped her to acquire new critical thinking strategies, which was one of her primary learning goals throughout this process.

Learning Outcome	Examples From Interview Data
Fundamental Research Skills	- Problem solving strategies, learning how to search the scientific literature, setting up a workspace (e.g. chemical hood with Schlenk line)
Techniques	- Synthetic organic research techniques including column chromatography, using thin layer chromatography (TLC) to monitor a reaction, and performing a sublimation
Increased Familiarity With Grad School	- What to expect in grad school, questions to ask and things to look for when choosing a graduate program and laboratory, “dos and do-nots”
Personal Growth	- Increase in confidence and independence, growth in identity as a learner and scientist

Table 4.3 – Learning Outcomes Perceived by Stanna from 10-Week Visit to the Montgomery Lab

According to the NRC’s *How People Learn*,[114] the transfer of knowledge to new settings and situations is an active learning strategy which is commonly used by experts who have a deep understanding of a particular subject matter and how it is applied in different contexts. When I interviewed Stanna two months after the program, she told me she had already started employing a few of these strategies to help advance her research in the Johnson lab. For example, Stanna mentioned she was now frequently utilizing the literature as a resource to troubleshoot problems and continuing to use TLC as a tool to monitor reaction conversion, both approaches she had not made use of before coming to the University of Michigan. Furthermore, Stanna’s visitation experience also gave her a much better understanding about graduate school and affirmed it was the right career path for her. She later recalled this being one of her primary motivations for wanting to conduct research at an external R1 institution:

“[G]oing into the summer I think the only expectation I had was that it would be a taste of grad school.” In addition, after working in the Montgomery lab Stanna began to consider issues related to graduate programs that she had not previously thought about such as graduation requirements, mentorship style of the professors, access to equipment, and location of the university.

Stanna also commented on the added benefits of being directly mentored by students who were significantly more experienced than most of her co-workers in the Johnson lab. While she recognized significant parallels between both laboratories’ mentorship practices, she acknowledged that the resources available to her in the Montgomery lab helped her to move beyond her unassisted capabilities in a way that was not possible in the Johnson lab. In this sense, Stanna was able to expand her zone of proximal development[96] beyond what was previously conceivable, highlighting a benefit of the exchange program. The experience also challenged her to move outside of her comfort zone by confronting difficult tasks that she often purposefully avoided in the Johnson lab. This allowed her to become more confident and independent in her research skills, which Stanna described as one of her primary goals for the summer:

> And so to be forced to learn how to do those things it’s really good for me and so while I feel like I definitely could have gotten better…at Hope, I definitely wouldn’t have gotten as comfortable as I am now [doing research] had I not come here.

As described earlier, Lave defined advancement in a community of practice as being not only correlated to an increase in knowledge and skills, but a change in social identity.[93,94] By the end of her research experience, Stanna not only felt that she had made a lot of professional connections, but sensed she had been fully apprenticed into the lab family: “But what I love about the lab is you guys are all so welcoming and you

all felt like my older siblings – like I'm the baby of the family and I just ask anybody questions. That was cool." In addition to identifying as a member of the Montgomery lab community, Stanna also discussed how she became more self-aware of her identity as a learner. As she mentioned in her second interview with me: "And I think…the more different personalities I see, the better I find out how I work." By becoming closely connected with Montgomery lab members, Stanna was able to relate to others and realize her own work-style preferences, consequently learning more about herself in the process.

4.5 Conclusions and Future Directions

Ultimately, we found our R1/PUI exchange program to be a worthwhile professional development experience which is well explained using situated theories of learning. By exchanging places in each other's communities of practice, Stanna and I experienced significant cognitive and personal gains that could not be duplicated if we had stayed place in our respective laboratories. While I learned important lessons on the importance of independence and enjoyment in students' laboratory learning, I also left the experience much happier than when I had started, having regained confidence and rediscovered my love for chemistry and teaching. Similarly, Stanna obtained a range of new skills and insights that are transferable to the next phase of her education. These lessons will not only help her to achieve success in her future research, but provide her with the confidence and determination to keep going on the days when this success may feel out of reach. In the end we feel these results support our claim that graduate/undergraduate exchange programs are relatively underexplored career

development opportunities that should be utilized more often to better prepare students for the next stages of their professional lives.

While I could not have acquired such detailed insight from simply visiting the Johnson laboratory, it is unclear how significantly these outcomes would be affected if a similar scenario were repeated in the absence of data collection and analysis. It would be unreasonable for us to suggest that future exchange program participants replicate these practices; however, at a very minimum we advise that students take full advantage of such opportunities by fully engaging with others and taking time for personal reflection. We also recommend that future research efforts examine students' outcomes from a fully external perspective (i.e. the researcher is not also a participant in the exchange program) and that studies focus on exchange programs in different settings and research areas to support more widespread understanding of and implementation of these experiences.

Logistical details and descriptions of learning gains from this study will be published in the near future, likely in a format which is easily accessible to the broader scientific community. In doing so, we hope that our work will not only inspire further research efforts but will promote the design of similar exchange program opportunities to better prepare students for careers in the sciences.

CHAPTER V

Supporting Information

5.1 General Experimental Details

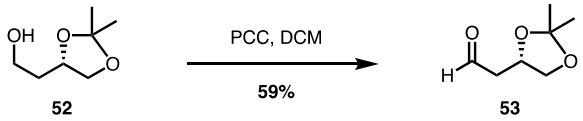
Unless otherwise noted, all reactions were conducted in flame- dried or oven-dried glassware with magnetic stirring under an atmosphere of dry nitrogen. Reaction temperatures were controlled using an IKA RCT basic stir plate with ETS-D5 electronic contact thermometer. Solvents were purified under nitrogen using a solvent purification system (Innovative Technology, Inc., Model #SPS- 400- 3 and PS- 400- 3). (+)-B-allyldiisopinocampheylborane,[117] and 2-((dimethylamino)methyl)benzoic acid,[62] and azidobenzoic acids[77] were synthesized according to previously reported procedures. Methacryloyl chloride (Sigma-Aldrich), Et_3N, and oxalyl chloride were distilled prior to use. (+)-*B*-Methoxydiisopinocampheylborane (Sigma-Aldrich), and Grubbs catalyst (2nd generation, Sigma-Aldrich) were stored and weighed in an inert atmosphere glovebox. Unless stated otherwise, all other chemical reagents were obtained from commercial sources and used as received.

Analytical thin layer chromatography (TLC) was performed on SiliaPlate TLC 60Å F-254 (250 μm silica gel) and compounds were visualized with ceric ammonium molybdate or aqueous KMnO4 stain. Flash column chromatography was performed using SiliaFlash® P60 (230- 400 mesh) silica gel.

1 H-Nuclear Magnetic Resonance (1 H-NMR) and 13 C-Nuclear Magnetic Resonance (13 C-NMR) spectra were recorded on Varian MR 400, Vnmrs 500, INOVA 500 and Vnmrs 700 MHz. NMR spectra were recorded in deuterated chloroform ($CDCl_3$) at room temperature. High-resolution mass spectra were recorded on a VG 70-250-s spectrometer manufactured by Micromass Corp. (Manchester UK) at the University of Michigan Mass Spectrometry Laboratory.

5.2 Synthesis of Tirandamycin Analogs

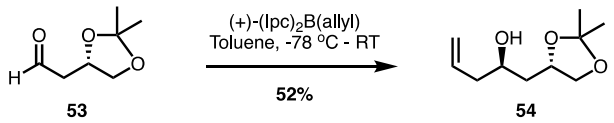
5.2.1 Bicycle Synthesis



52 53

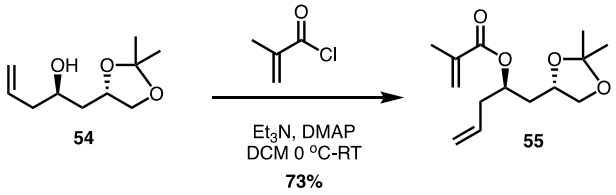
Scheme 2.9, Compound 53 ((*S*)-2-(2,2-dimethyl-1,3-dioxolan-4-yl)acetaldehyde):

(4*S*)-(+)-4-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxolane (**52**, 6.54 mL, 46.0 mmol, 1.0 equiv) was added to a flame-dried round bottom flask containing pyridinium chlorochromate (19.8 g, 92.0 mmol, 2.0 equiv), DCM (250 mL) and celite (20 g). The reaction was allowed to stir for approximately 2 hours and the resulting slurry filtered through a layered plug of celite and silica with ether. The filtrate was then slowly concentrated by rotary evaporation using an iced water bath to avoid loss of the volatile product. Residual chromium was removed via column chromatography (30 - 50% Et_2O/pentane) to give **53** (3.91 g, 27.1 mmol, 59%). The spectra matched that of previous reports.[118]



Scheme 2.9, Compound 54 ((R)-1-((S)-2,2-dimethyl-1,3-dioxolan-4-yl)pent-4-en-2-ol):

A solution of aldehyde **53** (2.72 g, 18.9 mmol, 1.0 equiv) in toluene (13 mL) was added dropwise to a cooled slurry of (+)-B-allyldiisopinocampheylborane[117] (7.39 g, 22.7 mmol,1.2 equiv) in toluene (20 mL) at -78 °C. The resulting mixture was stirred at -78 °C for 1.5 hours and then warmed to room temperature while stirring for an additional hour. The mixture was cooled to 0 °C and a premixed solution of 3M NaOH (36 mL), and 30% H_2O_2 (15 mL) was slowly added over 10 minutes using an addition funnel, followed by addition of saturated aq. $NaHCO_3$ (45 mL) over 3 minutes. The biphasic mixture was then allowed to stir for ~12 hours to hydrolyze borinate ester products. The organic and aqueous layers were separated and the aqueous layer was extracted twice with ether. The combined organic layers were then washed twice with brine and transferred to a round bottom flask. THF (85 mL), H_2O (45 mL), and iron (II) sulfate heptahydrate salt (8.5 g) were added and the biphasic mixture was stirred for another ~12 hours. The two layers were separated and the aqueous layer was extracted two times with ether. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated. Purification by column chromatography (10-30% EtOAc/Hexanes) afforded **54** (1.82 g, 9.76 mmol, 52%), which matched that of previous reports.[119]



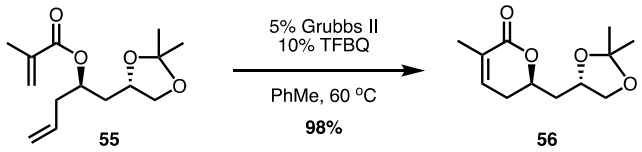
Scheme 2.9, Compound 55 ((*R*)-1-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)pent-4-en-2-yl methacrylate):

To a flame-dried round bottom flask equipped with a stir bar was added **54** (1.87 g, 10.0 mmol, 1.0 equiv), DMAP (612 mg, 5.01 mmol, 0.5 equiv), Et_3N (7.00 mL, 50.1 mmol, 5.0 equiv) and DCM (10 mL). The solution was cooled to 0 °C and methacryloyl chloride (1.96 mL, 20.0 mmol, 2.0 equiv) was slowly added. The resulting reaction mixture was allowed to warm to room temperature and stirred until completion, as judged by TLC. The mixture was then diluted with saturated aq. $NaHCO_3$ and brine, the organic layer separated, and the aqueous layer extracted three times with ether. The combined organic layers were dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography (10% EtOAc/Hexanes) to give the title compound (1.87 g, 7.35 mmol, 73%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.21 – 5.93 (m, 1H), 5.82 – 5.69 (m, 1H), 5.60 – 5.50 (m, 1H), 5.18 – 5.00 (m, 3H), 4.17 – 4.06 (m, 1H), 4.00 (dd, *J* = 8.0, 5.9 Hz, 1H), 3.52 (t, *J* = 7.6 Hz, 1H), 2.49 – 2.31 (m, 2H), 2.01 – 1.88 (m, 4H), 1.87 – 1.76 (m, 1H), 1.39 (s, 3H), 1.32 (s, 3H).

^{13}C NMR (176 MHz, $CDCl_3$): δ 166.74, 136.41, 133.07, 125.44, 118.24, 108.60, 73.34, 71.25, 69.76, 39.11, 37.85, 26.94, 25.70, 18.30.

HRMS (ESI+) *m/z*: $[M+Na]^+$ predicted for $C_{14}H_{22}O_4Na$, 277.1410; found 277.1410.



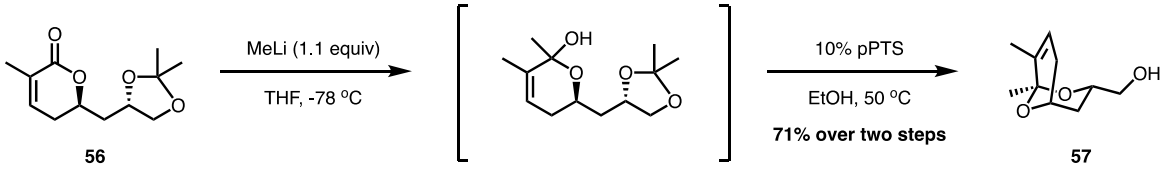
Scheme 2.9, Compound 56 ((*R*)-6-(((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)methyl)-3-methyl-5,6-dihydro-2*H*-pyran-2-one):

To a flame-dried three-necked flask equipped with a reflux condenser was added **55** (866 mg, 3.41 mmol, 1.0 equiv), tetrafluoro-1,4-benzoquinone (61.3 mg, 0.34 mmol, 0.1 equiv), and toluene (680 mL), followed by Grubbs' catalyst (145 mg, 0.17 mmol, 0.05 equiv). The reaction was heated for ~12 hours at 60 °C while sparging with N_2. A solution of Grubbs' catalyst (145 mg, 0.17 mmol, 0.05 equiv) was then added in 1 mL toluene and the reaction was allowed to stir for another ~2 hours or until complete conversion was observed by TLC. The resulting solution was cooled to room temperature, concentrated in vacuo and purified by column chromatography (7-30% EtOAc/Hexanes) to afford the final product (757 mg, 3.35 mmol, 98%).

^{1}H NMR (700 MHz, $CDCl_3$): δ 6.59 – 6.55 (m, 1H), 4.65 – 4.55 (m, 1H), 4.42 – 4.33 (m, 1H), 4.12 (dd, *J* = 8.1, 6.0 Hz, 1H), 3.55 (dd, *J* = 8.1, 7.1 Hz, 1H), 2.43 – 2.25 (m, 2H), 1.97 (ddd, *J* = 14.3, 9.4, 3.6 Hz, 1H), 1.93 – 1.89 (m, 3H), 1.81 (ddd, *J* = 14.2, 9.0, 3.3 Hz, 1H), 1.40 (s, 3H), 1.35 (s, 3H).

^{13}C NMR (176 MHz, $CDCl_3$): δ 165.66, 138.73, 128.53, 109.00, 75.38, 72.10, 69.55, 39.46, 30.37, 26.99, 25.61, 17.03.

HRMS (ESI+) *m/z*: $[M+H]^+$ predicted for $C_{12}H_{19}O_4$, 227.1278; found 277.1276.



56 57

Scheme 2.9, Compound 57 (((1R,3S,5R)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methanol):

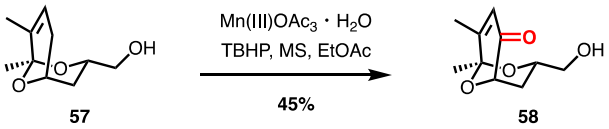
To a flame-dried round bottom flask was added a solution of **56** (206 mg, 0.91 mmol, 1.0 equiv) in THF (18 mL). The solution was cooled to -78 °C and then MeLi (626 μL, 1.6 M solution in Et_2O, 1.00 mmol) was slowly added. The resulting mixture was allowed to stir for 4 hours and then quenched by addition of saturated aq. NH_4Cl. The organic layer was then separated and the aqueous layer extracted three times with EtOAc. The combined organic extracts were dried with $MgSO_4$, filtered, concentrated, and used immediately in the next step without further purification.

The crude hemiketal and *p*-PTS (22.9 mg, 0.09 mmol, 0.1 equiv) were dissolved in EtOH (15 mL) and stirred for ~12 hours at 50 °C. The reaction was then quenched by addition of saturated aq. $NaHCO_3$. The organic layer was then separated and the aqueous layer extracted three times with Et_2O. The combined organic layers were then dried over $MgSO_4$, filtered, and concentrated in vacuo. The resulting crude product was purified by column chromatography (7-30% EtOAc/Hexanes) to give the title bicyclic ketal (118 mg, 0.64 mmol, 71% over two steps).

^{1}H NMR (500 MHz, $CDCl_3$): δ 5.84 – 5.67 (m, 1H), 4.34 (t, *J* = 6.6 Hz, 1H), 4.03 (ddt, *J* = 12.6, 6.3, 3.3 Hz, 1H), 3.64 – 3.55 (m, 1H), 3.54 – 3.45 (m, 1H), 2.73 – 2.58 (m, 1H), 2.10 – 2.03 (m, 1H), 2.03 – 1.95 (m, 1H), 1.93 – 1.85 (m, 1H), 1.67 – 1.61 (m, 3H), 1.42 (s, 3H), 1.24 (ddd, *J* = 13.3, 3.2, 1.2 Hz, 1H).

^{13}C NMR (176 MHz, $CDCl_3$): δ 132.69, 123.76, 95.59, 68.31, 66.28, 66.16, 31.55, 30.39, 24.66, 18.74.

HRMS (ESI+) *m/z*: $[M+H]^+$ predicted for $C_{10}H_{16}O_3$, 185.1172; found 185.1170.



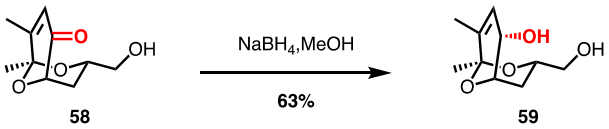
Scheme 2.10, Compound 58 ((1*S*,3*S*,5*S*)-3-(hydroxymethyl)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-6-one):

To a flame-dried round bottom flask under a nitrogen atmosphere was added **57** (102 mg, 0.56 mmol, 1.0 equiv), 3 Å molecular sieves (530 mg), and anhydrous EtOAc (6 mL). *tert*-Butyl hydroperoxide (1.04 mL, 5-6 M solution in decane, 5.71 mmol) was then slowly added and the resulting mixture was allowed to stir for 30 minutes at room temperature. Manganese (III) acetate dihydrate (29.7 mg, 0.11 mmol, 0.2 equiv) was added as a solution in EtOAc (~1 mL) and the reaction was stirred for 48 hours, or until complete, as judged by TLC. The mixture was then filtered through celite, concentrated, and purified by column chromatography (17-70% EtOAc/Hexanes) to afford the allylic ketone (49.2 mg, 0.25 mmol, 45%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.17 (s, 1H), 4.35 (d, *J* = 6.0 Hz, 1H), 3.90 (ddt, *J* = 12.3, 6.0, 3.1 Hz, 1H), 3.63 (dd, *J* = 11.9, 3.1 Hz, 1H), 3.54 (dd, *J* = 11.8, 5.6 Hz, 1H), 2.09 (m, 1H), 1.97 (d, *J* = 1.6 Hz, 3H), 1.91 (s, 1H), 1.55 (s, 3H), 1.50 (ddd, *J* = 13.7, 3.1, 1.5 Hz, 1H).

^{13}C NMR (126 MHz, $CDCl_3$): δ 196.91, 156.59, 127.09, 96.21, 74.49, 68.77, 65.61, 27.92, 24.77, 19.59.

HRMS (ESI+) *m/z*: $[M+NH_4]^+$ predicted for $C_{10}H_{18}O_4N$, 216.1230; found 216.1231.



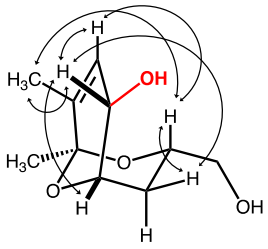
58 59

Scheme 2.10, Compound 59 (1*S*,3*S*,5*S*,6*S*)-3-(hydroxymethyl)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-6-ol:

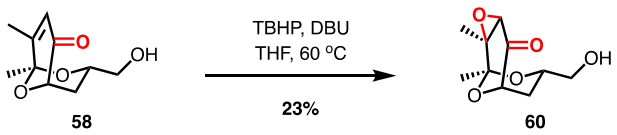
A solution of **58** (14.8 mg, 0.08 mmol, 1.0 equiv) in MeOH (1 mL) and cooled to 0 °C. $NaBH_4$ (8.50 mg, 0.22 mmol, 3.0 equiv) was added and the reaction was allowed to stir until completion, as judged by TLC. Amberlite 120 ion exchange resin was then added and allowed to stir for ~10 minutes. The mixture was filtered with MeOH over celite and concentrated in vacuo. The residue was then co-evaporated 3 times with MeOH and purified by column chromatography to give a single diastereomer (9.40 mg, 0.05 mmol, 63%).

^{1}H NMR (700 MHz, $CDCl_3$): δ 5.71 (s, 1H), 4.67 – 4.58 (m, 1H), 4.20 (t, *J* = 6.4 Hz, 1H), 3.97 (ddt, *J* = 9.5, 6.4, 3.7 Hz, 1H), 3.62 (dd, *J* = 11.6, 3.2 Hz, 1H), 3.52 (dd, *J* = 11.6, 6.1 Hz, 1H), 2.01 (s, 1H), 1.87 – 1.74 (m, 3H), 1.69 – 1.61 (m, 3H), 1.41 (s, 3H).

^{13}C NMR (176 MHz, $CDCl_3$): δ 134.52, 128.12, 95.59, 69.72, 68.49, 66.13, 65.60, 24.50, 23.30, 18.16.



59
Select NOESY Correlations



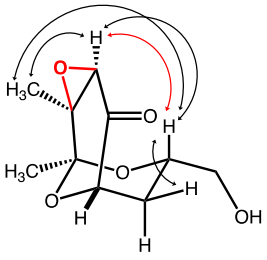
Scheme 2.10, Compound 60 (1*S*,2*S*,4*R*,6*S*,8*S*)-8-(hydroxymethyl)-1,2-dimethyl-3,9,10-trioxatricyclo[4.3.1.0^{2,4}]decan-5-one:

To a flame-dried reaction tube equipped with a stir bar was added a solution of **58** (7.7 mg, 0.04 mmol, 1.0 equiv) in THF (0.5 mL) under nitrogen. DBU (31.0 μL, 0.21 mmol, 5.3 equiv) was then added, followed by *tert*-Butyl hydroperoxide (37.8 μL, 5.5 M solution in decane, 0.21 mmol), and the resulting reaction mixture was heated to 60 °C for ~12 hours. The reaction vessel was cooled to room temperature and the mixture diluted with DCM and washed with 1M HCl. The aqueous layer was extracted with DCM and the combined organic layers washed with H_2O. The aqueous layer was extracted again with DCM and the combined organic layers dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography (50-70% EtOAc/Hexanes) afforded the final product (1.90 mg, 0.01 mmol, 23%).

^{1}H NMR (700 MHz, $CDCl_3$): δ 4.38 – 4.33 (m, 1H), 4.04 – 3.97 (m, 1H), 3.62 (dd, *J* = 11.8, 3.1 Hz, 1H), 3.52 (dd, *J* = 11.8, 5.9 Hz, 1H), 3.38 (s, 1H), 2.08 – 1.99 (m, 1H), 1.58 – 1.54 (m, 4H), 1.50 (s, 3H).

^{13}C NMR (176 MHz, $CDCl_3$): δ 205.31, 97.27, 74.48, 68.74, 65.69, 62.11, 58.42, 29.56, 23.09, 15.89.

HRMS (ESI+) *m/z*: $[M+H]^+$ predicted for $C_{10}H_{15}O_5$, 215.0914; found 215.0918.



60
Select NOESY Correlations

5.2.2 Anchor Attachment

General DCC Coupling Procedure:

To a flame-dried reaction vessel was added tirandamycin bicycle (1.0 equiv), benzoic acid (1.5 equiv), DCC (1.5 equiv), DMAP (0.15 equiv) and DCM (0.1M). The resulting mixture was allowed to stir for ~30 minutes, or until complete as judged by TLC. The crude mixture was then filtered twice through cotton and purified by column chromatography.

General EDC Coupling Procedure:

To a flame-dried reaction vessel was added tirandamycin bicycle (1.0 equiv), azidobenzoic acid[77] (5.0 equiv), EDC•HCl (5.0 equiv), DMAP (1.0 equiv) and DCM (0.05M). The resulting mixture was allowed to stir until complete as judged by TLC. The crude mixture was then concentrated and purified by column chromatography.

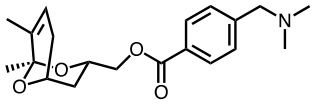
General Reductive Amination Procedure:

To a mixture of aldehyde (1.0 equiv) in DCM (0.15M) was added dimethyl amine hydrochloride (1.3 equiv) and the resulting reaction mixture was allowed to stir for ~30 minutes. The solution was then cooled to 0 ^{o}C and $NaBH(OAc)_3$ (1.5 equiv) was slowly added and stirred for ~12 hours. The reaction mixture was diluted with EtOAc and washed twice with saturated aq. $NaHCO_3$ and then once with 3:1 NaCl:$NaHCO_3$ solution. The organic phase was then dried over $MgSO_4$, filtered, and concentrated to yield a mixture of amine and alcohol. The resulting residue was then dissolved in 0.1M HCl and washed three times with 30% EtOAc/Hexanes. The aqueous layer was basified with saturated aq. $NaHCO_3$ until the pH increased to 8-9 and then extracted

three times with DCM. The combined organic layers were dried with Na_2SO_4, filtered and concentrated in vacuo.

General Click Cyclization Procedure:

Azido ester (1.0 equiv) and alkyne (1.0 equiv) were dissolved a 1:1 solution of H_2O/*t*-BuOH. Sodium ascorbate (10 mol%) and $CuSO_4 \cdot 5H_2O$ (1 mol%) were then added sequentially and the reaction was allowed to stir until the starting material was consumed as judged by TLC. Upon completion, the reaction was quenched with saturated aq. $NaHCO_3$ and the aqueous layer extracted five times with DCM. The organic layers were then combined, dried over $NaSO_4$, filtered, and concentrated. The crude residue was purified by column chromatography to yield the desired substrate.



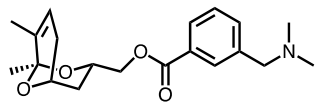
81

Figure 3.3/3.6, Compound 81 ((1R,3S,5R)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 4-((dimethylamino)methyl)benzoate:

Following the general DCC coupling procedure, the reaction of 23.0 mg (0.13 mmol) of **57**, 28.1 mg (0.19 mmol) of 4-formyl benzoic acid, 38.6 mg (0.19 mmol) of DCC, 2.30 mg (0.02 mmol) of DMAP, and 1.3 mL of DCM afforded 33.2 mg (0.10 mmol, 84%) of the desired aryl ester, which was purified by column chromatography (15% EtOAc/Hexanes) and carried on directly to the next step.

Following the general reductive amination procedure, the reaction of 33.2 mg (0.11 mmol) of ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 4-formylbenzoate, 11.1 mg (0.14 mmol) of dimethyl amine hydrochloride, 33.4 mg (0.16 mmol) $NaBH(OAc)_3$, and 1 mL of DCM afforded 10.9 mg (0.03 mmol, 30%) of the title compound.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.05 – 7.94 (m, 2H), 7.43 – 7.34 (m, 2H), 5.83 – 5.76 (m, 1H), 4.37 (t, *J* = 6.7 Hz, 1H), 4.33 – 4.24 (m, 3H), 3.47 (s, 2H), 2.76 – 2.62 (m, 1H), 2.24 (s, 6H), 2.09 – 1.97 (m, 1H), 1.97 – 1.87 (m, 1H), 1.70 – 1.62 (m, 3H), 1.45 (s, 3H), 1.44 – 1.39 (m, 1H).



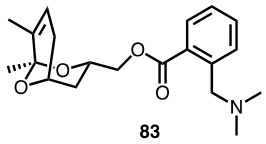
82

Figure 3.3/3.6, Compound 82 ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 3-((dimethylamino)methyl)benzoate:

Following the general DCC coupling procedure, the reaction of 31.3 mg (0.17 mmol) of **57**, 38.2 mg (0.25 mmol) of 3-formyl benzoic acid, 52.5 mg (0.25 mmol) of DCC, 3.10 mg (0.03 mmol) of DMAP, and 1.8 mL of DCM afforded 29.1 mg (0.09 mmol, 54%) of the desired aryl ester, which was purified by column chromatography (15% EtOAc/Hexanes) and carried on directly to the next step.

Following the general reductive amination procedure, the reaction of 29.1 mg (0.09 mmol) of ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 3-formylbenzoate, 9.75 mg (0.12 mmol) of dimethyl amine hydrochloride, 29.2 mg (0.14 mmol) $NaBH(OAc)_3$, and 1 mL of DCM afforded 14.7 mg (0.04 mmol, 46%) of the title compound.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.98 – 7.89 (m, 2H), 7.58 – 7.48 (m, 1H), 7.40 (t, *J* = 7.6 Hz, 1H), 5.84 – 5.75 (m, 1H), 4.37 (t, *J* = 6.6 Hz, 1H), 4.33 – 4.25 (m, 3H), 3.47 (s, 2H), 2.75 – 2.62 (m, 1H), 2.25 (s, 6H), 2.11 – 1.98 (m, 1H), 1.95 – 1.87 (m, 1H), 1.71 – 1.63 (m, 3H), 1.45 (s, 3H), 1.44 – 1.39 (m, 1H).

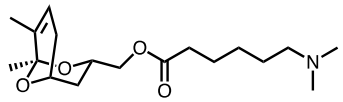


83

Figure 3.3/3.6, Compound 83 ((1R,3S,5R)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 2-((dimethylamino)methyl)benzoate:

To a flame-dried 1-dram vial was added 2-((dimethylamino)methyl)benzoic acid[62] (43.0 mg, 0.24 mmol, 1.0 equiv), followed by 3 drops of DMF and oxalyl chloride (23.0 μL, 0.27 mmol, 1.1 equiv). The reaction mixture was allowed to stir at room temperature for 1.5 hours and then concentrated in vacuo and resuspended in DCM (1.5 mL). The acyl chloride suspension was then added dropwise to an iced solution of **57** (50.0 mg, 0.27 mmol, 1.1 equiv), DMAP (14.7 mg, 0.12 mmol, 0.5 equiv), and Et_3N (168 μL, 1.20 mmol, 5.0 equiv) in DCM (1.8 mL). The mixture was allowed to stir until completion, with addition of more acyl chloride if necessary. The reaction was quenched with saturated aq. $NaHCO_3$ and washed three times with EtOAc. The combined organic layers were then dried with sodium sulfate, filtered, and concentrated to give the title compound (26.4 mg, 0.078 mmol, 32%) following purification by column chromatography.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.80 (d, *J* = 7.7 Hz, 1H), 7.54 – 7.39 (m, 2H), 7.31 (t, *J* = 7.5 Hz, 1H), 5.83 – 5.74 (m, 1H), 4.37 (t, *J* = 6.6 Hz, 1H), 4.33 – 4.19 (m, 3H), 3.79 (q, *J* = 13.6 Hz, 2H), 2.76 – 2.59 (m, 1H), 2.26 (s, 6H), 2.02 (td, *J* = 12.4, 6.2 Hz, 1H), 1.96 – 1.86 (m, 1H), 1.66 (s, 3H), 1.44 (s, 3H), 1.42 – 1.37 (m, 1H).



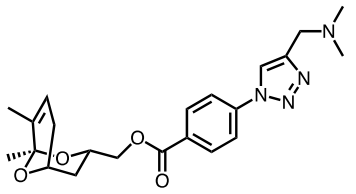
84

Figure 3.3/3.6, Compound 84 ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 6-(dimethylamino)hexanoate:

Following the general DCC coupling procedure, the reaction of 31.3 mg (0.17 mmol) of **57**, 49.6 mg (0.25 mmol) of 6-bromohexanoic acid, 52.5 mg (0.25 mmol) of DCC, 3.10 mg (0.03 mmol) of DMAP, and 1.8 mL of DCM afforded 26.4 mg (0.07 mmol, 43%) of the desired bromo ester, which was purified by column chromatography and carried on directly to the next step.

To a solution of ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 6-bromohexanoate (26.4 mg, 0.07 mmol, 1.0 equiv) in DMF (1 mL) was added dimethylamine (48 μL, 40 % w/w in H_2O, 0.38 mmol) and the resulting mixture was allowed to stir for ~2 hours. The reaction was then quenched with saturated aq. $NaHCO_3$ solution and extracted three times with EtOAc. The organic layers were combined, dried over Na_2SO_4, and concentrated in vacuo. Purification by column chromatography afforded the final product (14.9 mg, 0.05 mmol, 63%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.86 – 5.67 (m, 1H), 4.34 (t, *J* = 6.6 Hz, 1H), 4.21 – 3.96 (m, 3H), 2.75 – 2.56 (m, 1H), 2.39 – 2.28 (m, 2H), 2.27 – 2.14 (m, 8H), 1.98 – 1.82 (m, 2H), 1.71 – 1.57 (m, 5H), 1.53 – 1.44 (m, 2H), 1.42 (s, 3H), 1.39 – 1.27 (m, 3H).



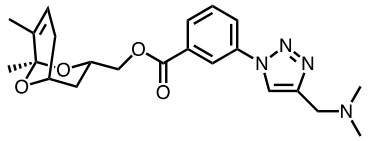
87

Figure 3.3/3.6, Compound 87 ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 4-(4-((dimethylamino)methyl)-1*H*-1,2,3-triazol-1-yl)benzoate:

Following the general EDC coupling procedure, the reaction of 30.0 mg (0.16 mmol) of **57**, 133 mg (0.81 mmol) of 4-azidobenzoic acid, 156 mg (0.81 mmol) of EDC•HCl, 19.9 mg (0.16 mmol) of DMAP, and 3.3 mL of DCM afforded 44.4 mg (0.13 mmol, 83%) of the desired azido ester, which was purified by column chromatography (0 to 10% EtOAc/Hexanes) and carried on directly to the next step.

Following the general click cyclization procedure, the reaction of 44.4 mg (0.13 mmol) of ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 4-azidobenzoate, 15 μL (0.14 mmol) *N*,*N*-dimethylprop-2-yn-1- amine, 10.0 x 10^{-3} mmol sodium ascorbate, 1.00 x 10^{-3} mmol $CuSO_4$•$5H_2O$, and 1 mL H_2O/*t*-BuOH (1:1) afforded 38.7 mg (0.09 mmol, 69%) of the title compound after purification by column chromatography.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.25 – 8.14 (m, 2H), 8.01 (s, 1H), 7.89 – 7.78 (m, 2H), 5.83 – 5.77 (m, 1H), 4.38 (t, *J* = 6.6 Hz, 1H), 4.35 – 4.27 (m, 3H), 3.69 (s, 2H), 2.75 – 2.62 (m, 1H), 2.32 (s, 6H), 2.11 – 1.98 (m, 1H), 1.98 – 1.88 (m, 1H), 1.70 – 1.62 (m, 3H), 1.45 (s, 3H), 1.43 – 1.38 (m, 1H).



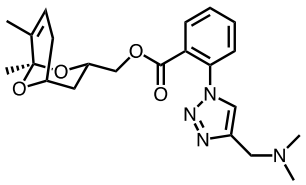
88

Figure 3.3/3.6, Compound 88 ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 3-(4-((dimethylamino)methyl)-1*H*-1,2,3-triazol-1-yl)benzoate:

Following the general EDC coupling procedure, the reaction of 30.0 mg (0.16 mmol) of **57**, 133 mg (0.81 mmol) of 3-azidobenzoic acid, 156 mg (0.81 mmol) of EDC•HCl, 19.9 mg (0.16 mmol) of DMAP, and 3.3 mL of DCM afforded 48.5 mg (0.15 mmol, 90%) of the desired azido ester, which was purified by column chromatography and carried on directly to the next step.

Following the general click cyclization procedure, the reaction of 48.5 mg (0.15 mmol) of ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 3-azidobenzoate, 16 μL (0.15 mmol) *N*,*N*-dimethylprop-2-yn-1- amine, 10.0 x 10^{-3} mmol sodium ascorbate, 1.00 x 10^{-3} mmol $CuSO_4$•$5H_2O$, and 1 mL H_2O/*t*-BuOH (1:1) afforded 39.2 mg (0.09 mmol, 65%) of the title compound after purification by column chromatography.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.38 – 8.29 (m, 1H), 8.13 – 8.06 (m, 1H), 8.05 – 7.96 (m, 2H), 7.61 (t, *J* = 7.9 Hz, 1H), 5.83 – 5.76 (m, 1H), 4.37 (t, *J* = 6.6 Hz, 1H), 4.35 – 4.28 (m, 3H), 3.70 (s, 2H), 2.75 – 2.60 (m, 1H), 2.33 (s, 6H), 2.09 – 1.96 (m, 1H), 1.96 – 1.87 (m, 1H), 1.70 – 1.60 (m, 3H), 1.47 – 1.38 (m, 4H).



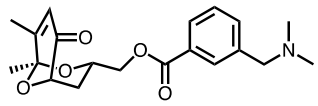
89

Figure 3.3/3.6, Compound 89 ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 2-(4-((dimethylamino)methyl)-1*H*-1,2,3-triazol-1-yl)benzoate:

Following the general EDC coupling procedure, the reaction of 30.0 mg (0.16 mmol) of **57**, 133 mg (0.81 mmol) of 2-azidobenzoic acid, 156 mg (0.81 mmol) of EDC•HCl, 19.9 mg (0.16 mmol) of DMAP, and 3.3 mL of DCM afforded 43.9 mg (0.13 mmol, 82%) of the desired azido ester, which was purified by column chromatography and carried on directly to the next step.

Following the general click cyclization procedure, the reaction of 43.9 mg (0.13 mmol) of ((1*R*,3*S*,5*R*)-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 2-azidobenzoate, 14 μL (0.13 mmol) *N*,*N*-dimethylprop-2-yn-1- amine, 10.0 x 10^{-3} mmol sodium ascorbate, 1.00 x 10^{-3} mmol $CuSO_4$•$5H_2O$, and 1 mL H_2O/*t*-BuOH (1:1) afforded 41.2 mg (0.10 mmol, 75%) of the title compound after purification by column chromatography.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.03 – 7.94 (m, 1H), 7.76 (s, 1H), 7.69 – 7.62 (m, 1H), 7.62 – 7.55 (m, 1H), 7.51 – 7.43 (m, 1H), 5.79 – 5.73 (m, 1H), 4.30 (t, *J* = 6.6 Hz, 1H), 4.15 – 4.08 (m, 2H), 4.08 – 4.00 (m, 1H), 3.70 (s, 2H), 2.70 – 2.56 (m, 1H), 2.33 (s, 6H), 1.94 – 1.77 (m, 2H), 1.66 – 1.57 (m, 3H), 1.39 (s, 3H), 1.31 – 1.20 (m, 1H).



92

Figure 3.5/3.6, Compound 92 ((1S,3S,5S)-1,8-dimethyl-6-oxo-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 3-((dimethylamino)methyl)benzoate:

Following the general DCC coupling procedure, the reaction of 14.9 mg (0.08 mmol) of **58**, 16.9 mg (0.11 mmol) of 3-formyl benzoic acid, 23.3 mg (0.11 mmol) of DCC, 1.40 mg (0.01 mmol) of DMAP, and 1.0 mL of DCM afforded 15.7 mg (0.05 mmol, 63%) of the desired aryl ester, which was purified by column chromatography and carried on directly to the next step.

Following the general reductive amination procedure, the reaction of 15.7 mg (0.05 mmol) of ((1*S*,3*S*,5*S*)-1,8-dimethyl-6-oxo-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 3-formylbenzoate, 5.00 mg (0.06 mmol) of dimethyl amine hydrochloride, 15.1 mg (0.71 mmol) $NaBH(OAc)_3$, and 1 mL of DCM afforded 7.30 mg (0.02 mmol, 42%) of the title compound.

^{1}H NMR (700 MHz, $CDCl_3$): δ 7.96 – 7.93 (m, 1H), 7.91 (d, *J* = 7.8 Hz, 1H), 7.55 (d, *J* = 7.6 Hz, 1H), 7.41 (t, *J* = 7.6 Hz, 1H), 6.20 (s, 1H), 4.39 (d, *J* = 6.0 Hz, 1H), 4.34 (dd, *J* = 11.5, 5.7 Hz, 1H), 4.29 (dd, *J* = 11.5, 4.5 Hz, 1H), 4.21 – 4.13 (m, 1H), 3.47 (s, 2H), 2.25 (s, 6H), 2.11 (td, *J* = 13.1, 6.3 Hz, 1H), 1.98 (d, *J* = 1.4 Hz, 3H), 1.69 (ddd, *J* = 13.6, 3.1, 1.6 Hz, 1H), 1.57 (s, 3H).

^{13}C NMR (176 MHz, $CDCl_3$): δ 196.58, 166.31, 156.53, 139.46, 134.02, 130.32, 129.83, 128.56, 128.53, 127.17, 96.25, 74.47, 66.82, 66.39, 63.93, 45.40, 29.04, 24.68, 19.52.

HRMS (ESI+) *m/z*: $[M+H]^+$ predicted for $C_{20}H_{26}NO_5$, 360.1805; found 360.1805.

5.3 Biochemical Oxidations

Preparation of PikC-RhFRED mutants:

PikC mutants were prepared following previously established protocols.[63,75]

Expression and purification of PikC-RhFRED and TamI-RhFRED:

The plasmid encoding the respective fusion protein was transformed into *E. coli* BL21(DE3) cells, and an individual colony was selected for overnight growth (37 °C) in 250 mL of LB containing kanamycin (50 μg/mL). 12 x 1.5 L of LB (2.8 L baffled Fernbach flasks) supplemented with kanamycin (50 μg/mL), thiamine (1 mM), and $FeCl_3$ (100 μM) were each inoculated with 15 mL of overnight seed culture and incubated at 37 °C (160 rpm). When the OD600 reached 0.6-1.0, the cultures were cooled in an ice-water bath (15-20 min) before addition of IPTG (0.1 mM) and δ-aminolevulinic acid (1 mM). The cultures were grown at 18 °C for 18-20 h before the cells were harvested and stored at -80 °C until used for protein purification. All subsequent steps were performed at 4 °C. The cells were thawed and resuspended in 180 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM PMSF, pH 8) prior to lysis via sonication. The crude lysate was centrifuged at 50,000 x g for 30 min to remove cellular debris, and the clarified lysate was incubated with 20 mL of pre-equilibrated Ni-NTA resin on a nutating shaker for 1-2 h. The slurry was loaded onto an empty column, and the lysate was pushed through with gentle syringe pressure. The resin was washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 8) to remove bulk protein contaminants prior to elution of PikC-RhFRED/TamI-RhFRED with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM

imidazole, 10% glycerol, pH 8). The protein was subsequently concentrated using 30-50 kD MWCO centrifugal filters and desalted by loading onto PD-10 columns and eluting with storage buffer (50 mM NaH_2PO_4, 1 mM EDTA, 0.2 mM DTT, 10% glycerol, pH 7.3). The purified enzyme was typically used right away for large-scale reactions; any unused enzyme was aliquoted, flash frozen in liquid N_2, and stored at -80 °C for subsequent use in analytical- and/or additional large-scale reactions.

Analytical-scale enzymatic reactions

Analytical-scale enzymatic reactions were carried out under the following conditions: 5 μM PikC-RhFRED/TamI-RhFRED, 1 mM substrate (5% DMSO, final concentration), 1 mM $NADP^+$, 5 mM glucose-6-phosphate, and 1 U/mL glucose-6-phosphate dehydrogenase in storage buffer (50 mM NaH_2PO_4 (pH = 7.3), 1 mM EDTA, 0.2 mM DTT, 10% (v/v) glycerol). The total volume of each reaction was 100 μL (carried out in a 1.7 mL Eppendorf tube), and reactions were incubated at 30 °C (200 rpm) for 14-18 h prior to quenching via extraction with chloroform (2 x 100 μL). The combined organic layers were dried under a gentle stream of N_2 and resuspended in methanol prior to LC-MS analysis. The subsequent liquid chromatography mass spectrometry (LC-MS) analysis was performed on an Agilent Q-TOF HPLC-MS (Department of Chemistry, University of Michigan) equipped with an high resolution electrospray mass spectrometry (ESI-MS) source and a Beckmann Coulter reverse-phase HPLC system using an Waters XBridge C18 3.5 μm, 2.1x150 mm under the following conditions: mobile phase (A = deionized water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid), 10% to 100% B over 15 min, 100% B for 4 min; flow rate, 0.2 mL/min. Reactions were scanned for [M+H] (starting material), [M+H+16] (monohydoxylation), [M+H+32]

(dihydroxylation), [M+H-14] (demethylated starting material), and [M+H+2] (demethylated monohydroxylated product). The percent conversion was determined as outlined in Li et al.[75] Briefly, the percent conversion was calculated with (AUCtotal products/(AUCtotal products + AUCunreacted substrate) by assuming ionization efficiency of substrate and hydroxylated products are the same, because the ionization site of this series of compounds should be the dimethylamino group.[75]

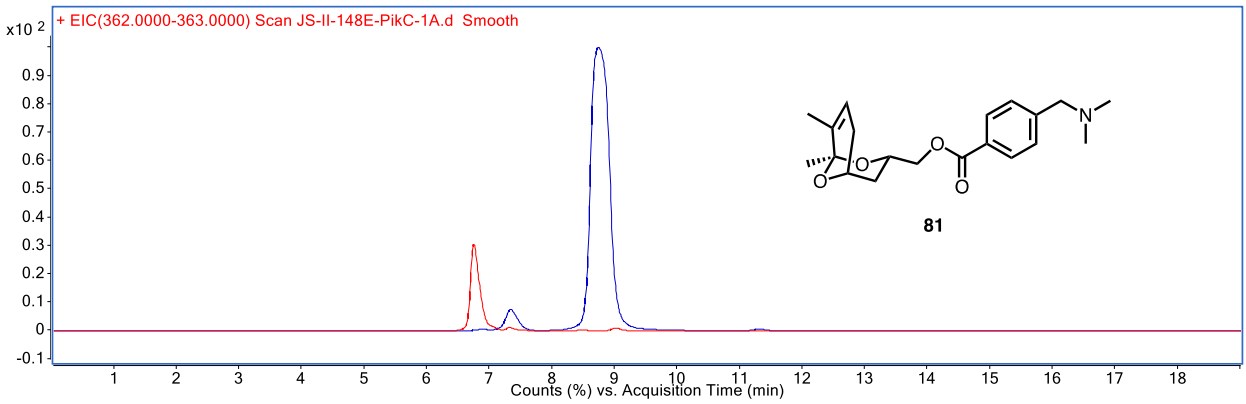
Preparative-scale enzymatic reactions

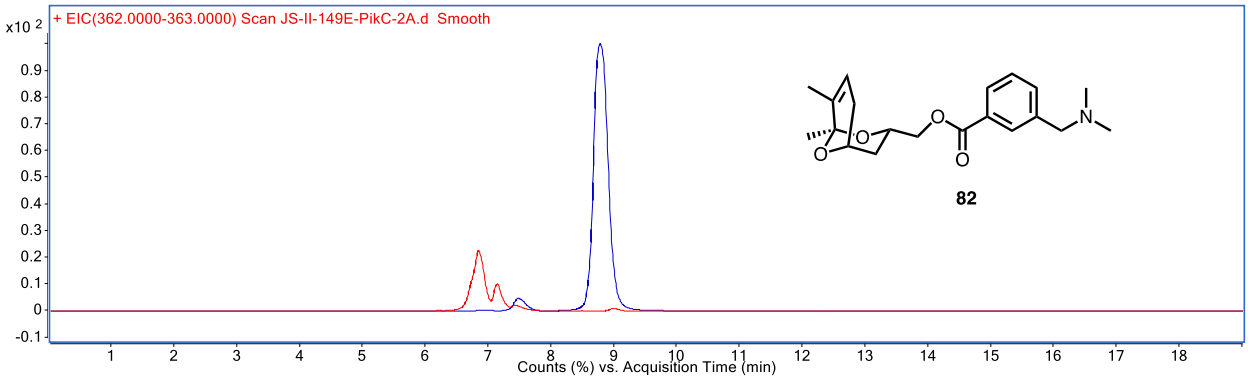
To an Erlenmeyer flask containing reaction buffer (50 mM NaH_2PO_4, 1 mM EDTA, 0.2 mM DTT, 10% (v/v) glycerol, pH 7.3) were added the following components sequentially: substrate (20 mM stock in DMSO, 1 mM final concentration), glucose-6-phosphate (100 mM stock in reaction buffer, 5 mM final concentration), glucose-6-phosphate dehydrogenase (100 U/mL stock in water, 1 U/mL final concentration), PikC-RhFRED/TamI-RhFRED (varied stock concentrations, 5 μM final concentration), and $NADP^+$ (20 mM stock in reaction buffer, 1 mM final concentration). The reaction mixture was capped with a milk filter and incubated at 30 °C overnight (14-16 h) with gentle shaking (100 rpm). Reactions were typically conducted on ~60 mg of each substrate (~130 mL total reaction volume) and performed in 500 mL Erlenmeyer flasks. After overnight incubation, the reaction was quenched by addition of acetone (2 x total reaction volume) and incubated at 4 °C for 2 h. The mixture was then filtered through celite, and the acetone was removed under reduced pressure. The remaining aqueous solution was saturated with NaCl, adjusted to pH 11, and extracted with ethyl acetate (3 x total reaction volume). The combined organic layers were dried over anhydrous Na2SO4 and evaporated under reduced pressure to afford a crude mixture of products

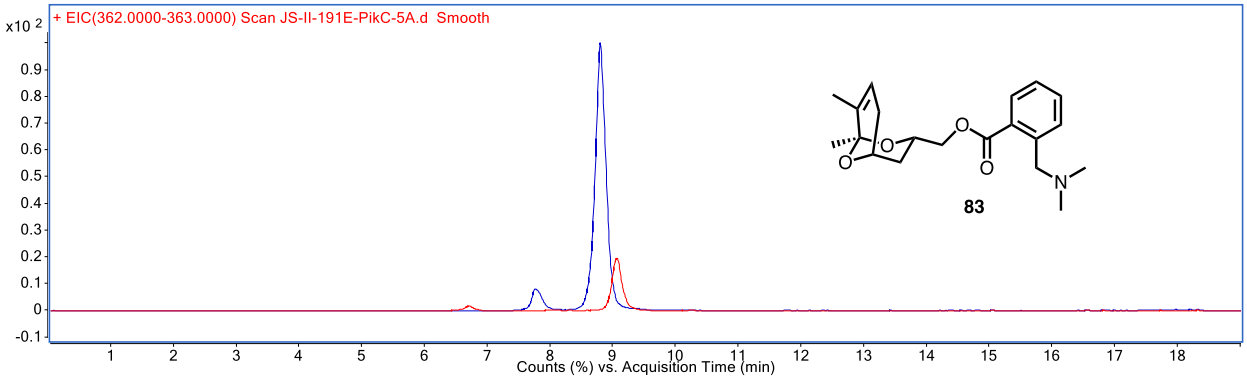
and remaining starting material, which were purified by flash column chromatography. Mixtures that were recalcitrant to separation using standard column chromatographic techniques were further purified via semi-preparative HPLC using a Beckman Coulter System Gold HPLC equipped with a Waters XBridge BEH C18 column (dimensions, 250 x 10 mm; particle size, 5 μm; pore size, 130 Å). The flow rate was maintained at 3 mL/min, and the mobile phase consisted of an acetonitrile/water mixture with formic acid (0.1%) included as a modifier. All crude material was dissolved in methanol and filtered through 0.20 μm PTFE filters (EMD Millipore) prior to manual HPLC injection. UV absorption was monitored at 240 nm and 260 nm.

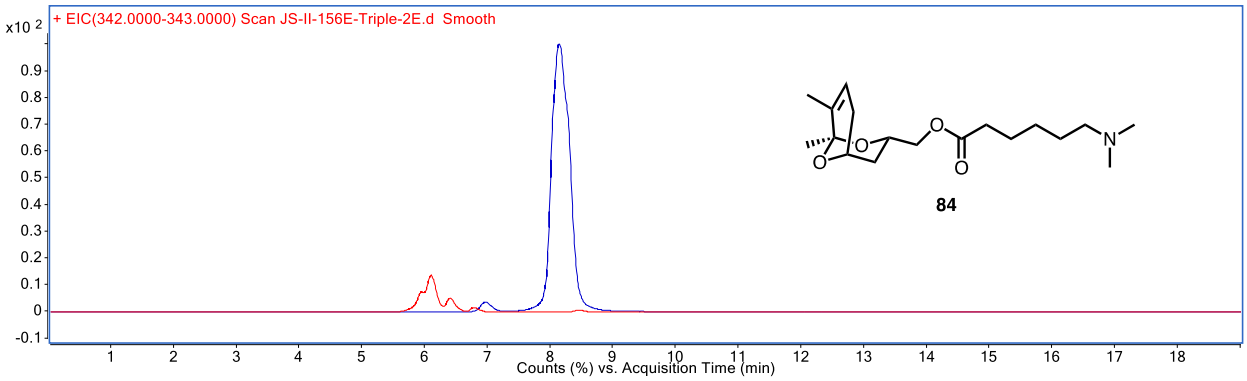
5.4 LCMS Traces

5.4.1 Reaction of Substrates with PikC$_{D50ND176QE246A}$-RhFRED (Figure 3.3)

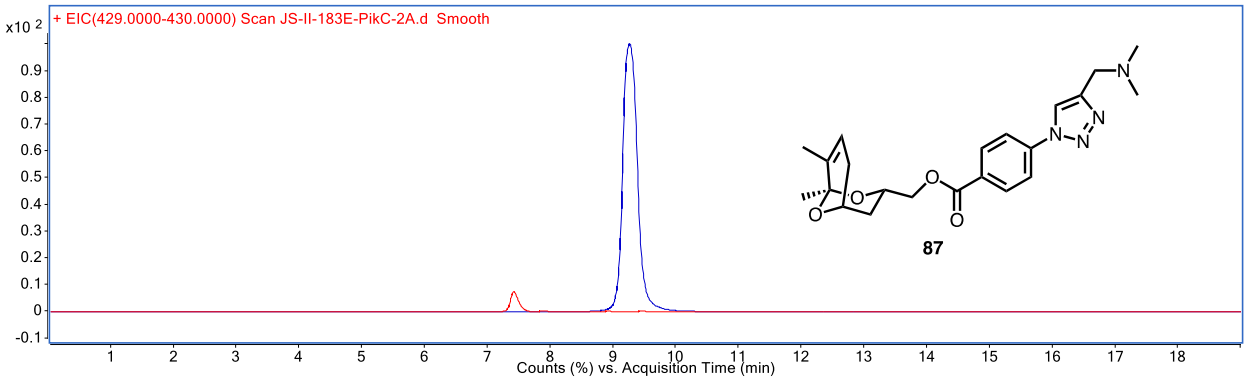




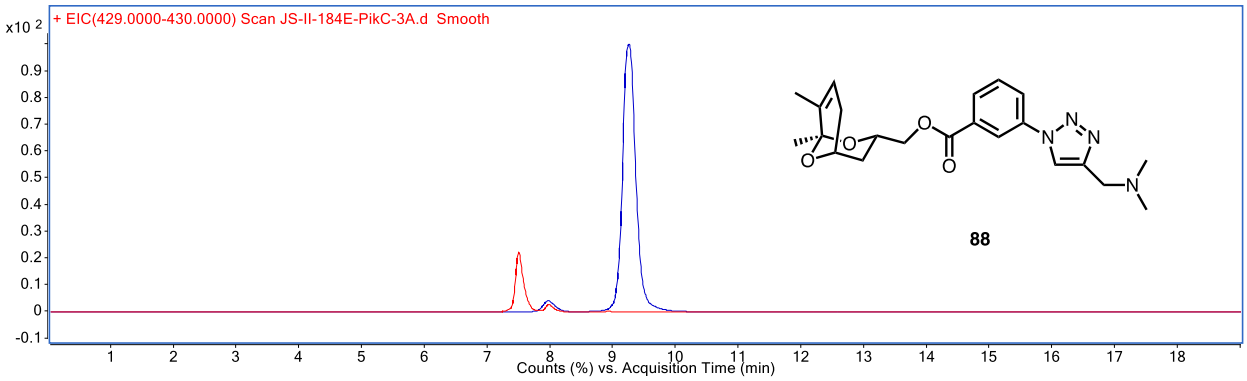



+ EIC(342.0000-343.0000) Scan JS-II-156E-Triple-2E.d Smooth
x10 2
Counts (%) vs. Acquisition Time (min)

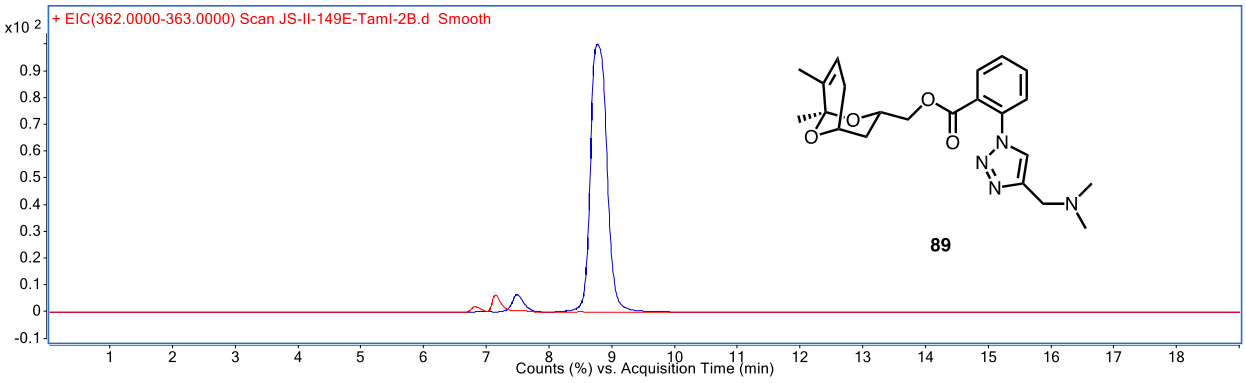
84


+ EIC(429.0000-430.0000) Scan JS-II-183E-PikC-2A.d Smooth
x10 2
Counts (%) vs. Acquisition Time (min)

87

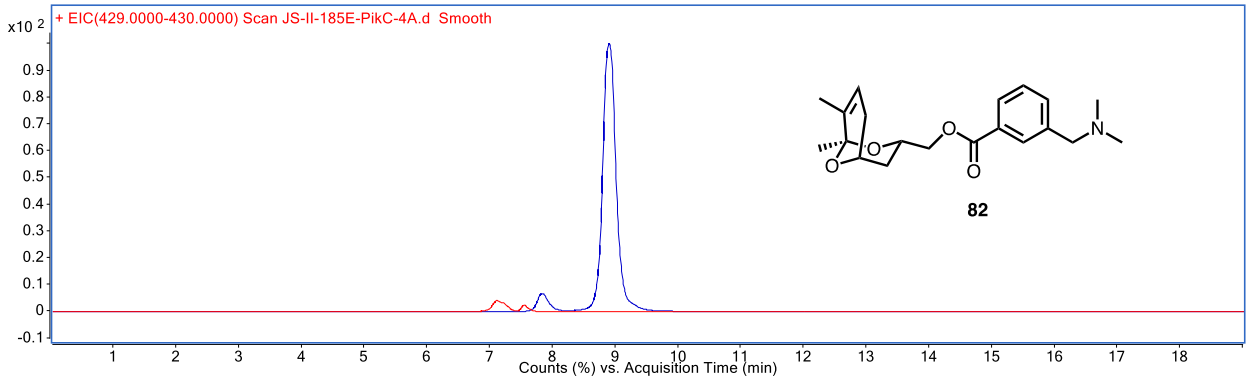

+ EIC(429.0000-430.0000) Scan JS-II-184E-PikC-3A.d Smooth
x10 2
Counts (%) vs. Acquisition Time (min)

88


+ EIC(362.0000-363.0000) Scan JS-II-149E-TamI-2B.d Smooth
x10 2
Counts (%) vs. Acquisition Time (min)

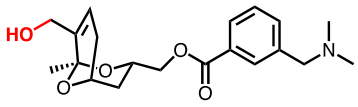
89

5.4.2 Reaction of **82** with TamI-RhFRED (Figure 3.6)



82

5.5 Enzymatic Product Characterization



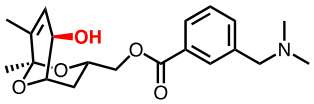
90

Figure 3.4, Compound 90 ((1*R*,3*S*,5*R*)-8-(hydroxymethyl)-1-methyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 3-((dimethylamino)methyl)benzoate:

^{1}H NMR (700 MHz, $CDCl_3$): δ 8.01 – 7.96 (m, 1H), 7.92 (dt, *J* = 7.8, 1.5 Hz, 1H), 7.51 (dt, *J* = 7.6, 1.4 Hz, 1H), 7.39 (t, *J* = 7.7 Hz, 1H), 6.20 – 6.16 (m, 1H), 4.49 – 4.44 (m, 1H), 4.41 (t, *J* = 6.5 Hz, 1H), 4.30 – 4.27 (m, 2H), 4.25 – 4.21 (m, 1H), 4.07 – 4.03 (m, 1H), 3.48 (d, *J* = 2.2 Hz, 2H), 2.79 – 2.71 (m, 1H), 2.25 (s, 6H), 2.07 – 2.00 (m, 2H), 1.52 (s, 3H), 1.43 (ddd, *J* = 13.2, 3.1, 1.3 Hz, 1H).

^{13}C NMR (176 MHz, $CDCl_3$): δ 166.58, 139.19, 135.22, 133.98, 130.54, 130.21, 128.65, 128.57, 125.95, 95.06, 67.59, 66.44, 65.96, 63.99, 63.40, 45.40, 32.25, 29.88, 24.45.

HRMS (ESI+) *m/z*: $[M+H]^+$ predicted for $C_{20}H_{28}NO_5$, 362.1962; found 362.1978.



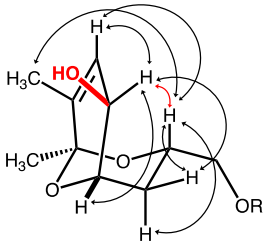
91

Figure 3.4, Compound 91 ((1*S*,3*S*,5*S*,6*S*)-6-hydroxy-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-3-yl)methyl 3-((dimethylamino)methyl)benzoate:

^{1}H NMR (700 MHz, $CDCl_3$): δ 7.96 – 7.94 (m, 1H), 7.94 – 7.92 (m, 1H), 7.55 (d, *J* = 7.6 Hz, 1H), 7.41 (t, *J* = 7.6 Hz, 1H), 6.00 – 5.97 (m, 1H), 4.31 (d, *J* = 7.0 Hz, 1H), 4.29 – 4.27 (m, 2H), 4.16 – 4.09 (m, 1H), 3.76 (d, *J* = 4.9 Hz, 1H), 3.48 (s, 2H), 2.25 (s, 6H), 2.06 – 1.97 (m, 1H), 1.75 – 1.68 (m, 3H), 1.50 (dd, *J* = 13.6, 3.3 Hz, 1H), 1.47 (d, *J* = 1.0 Hz, 3H).

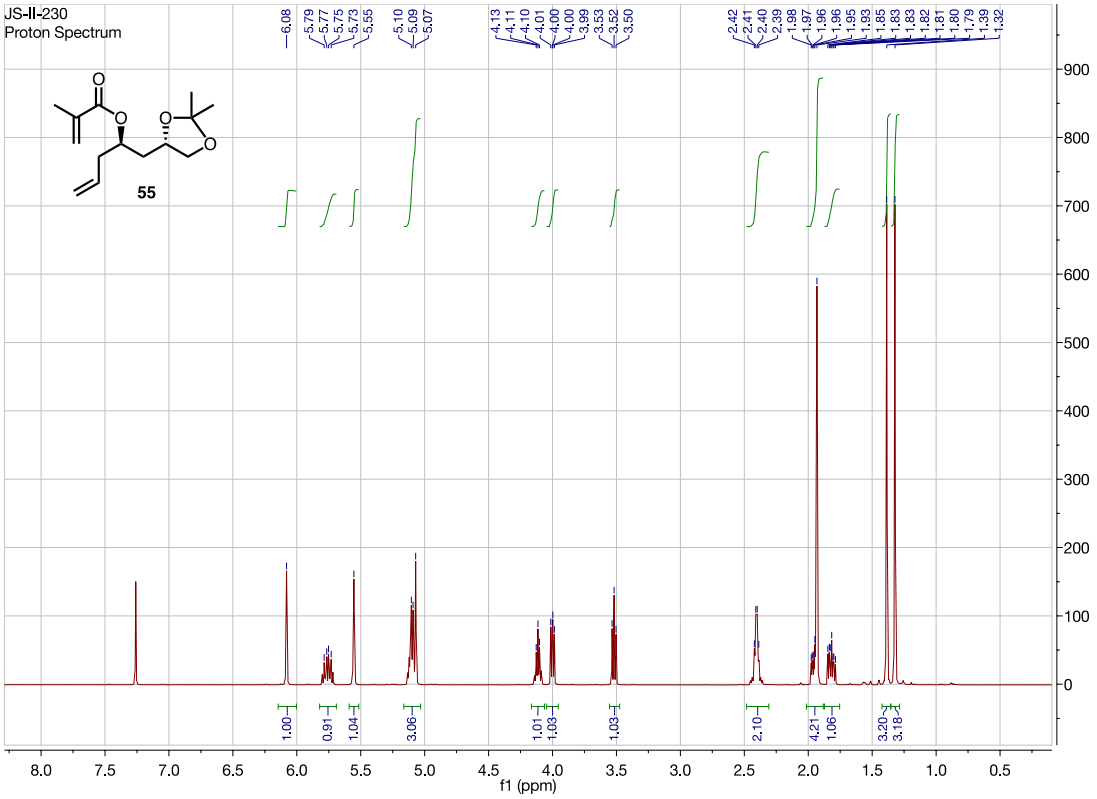
^{13}C NMR (176 MHz, $CDCl_3$): δ 166.58, 139.47, 136.83, 134.00, 130.42, 130.08, 128.63, 128.56, 125.56, 95.59, 73.90, 67.49, 66.35, 65.93, 64.01, 45.46, 28.67, 24.42, 18.62.

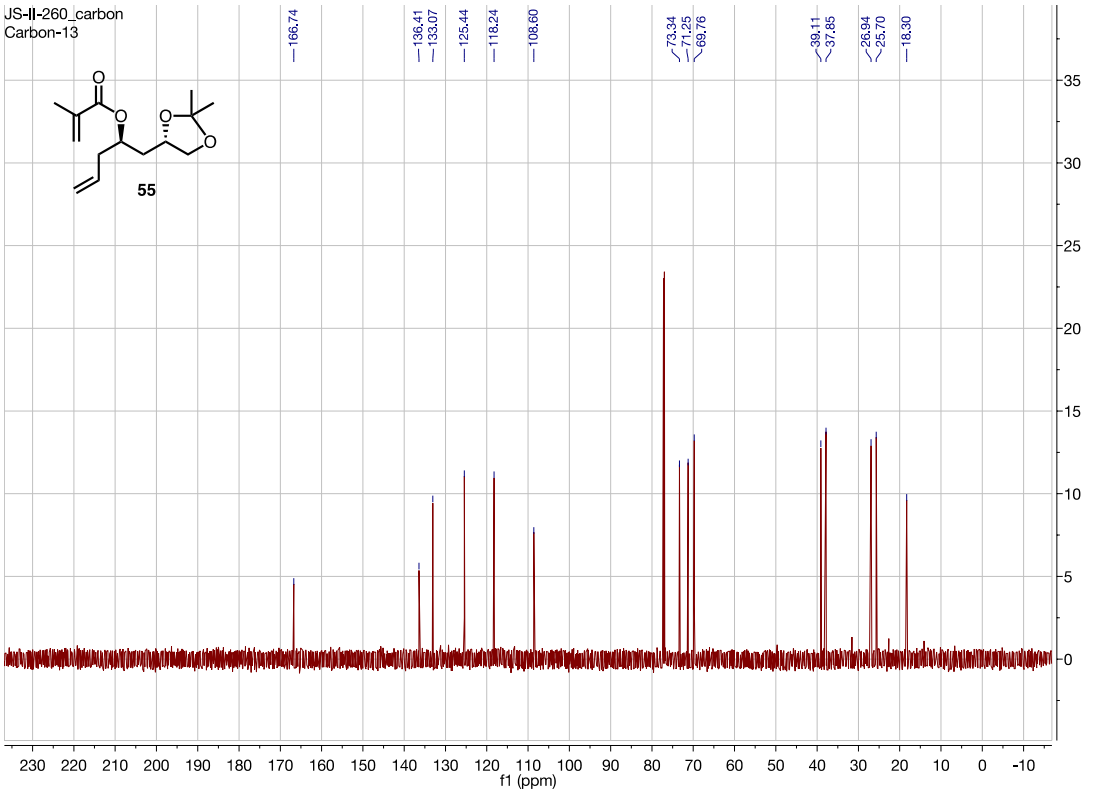
HRMS (ESI+) *m/z*: $[M+H]^+$ predicted for $C_{20}H_{28}NO_5$, 362.1962; found 362.1988.

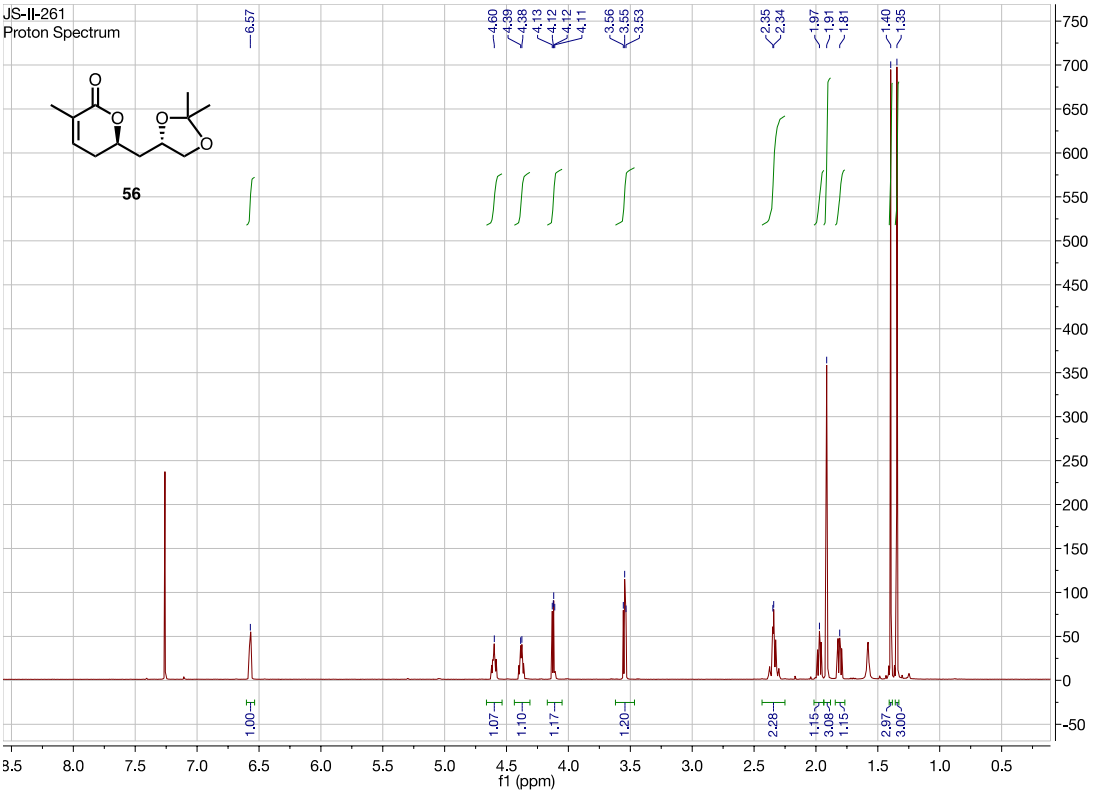


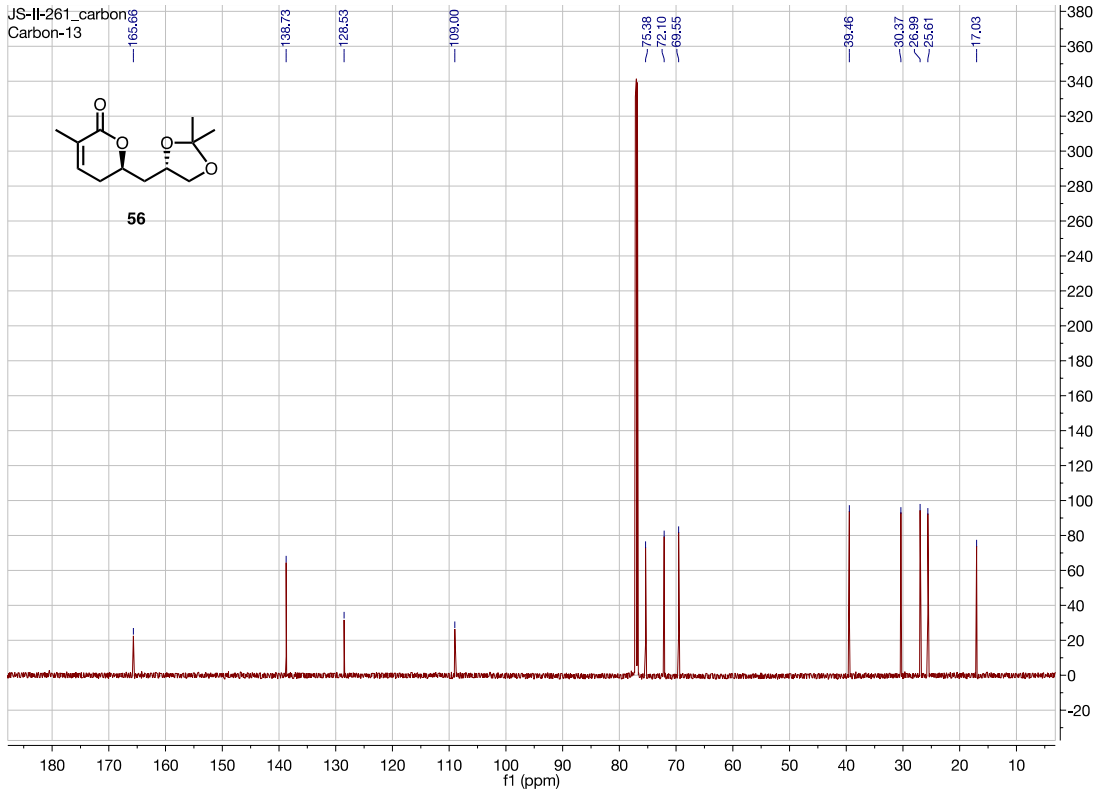
91
Select NOESY Correlations

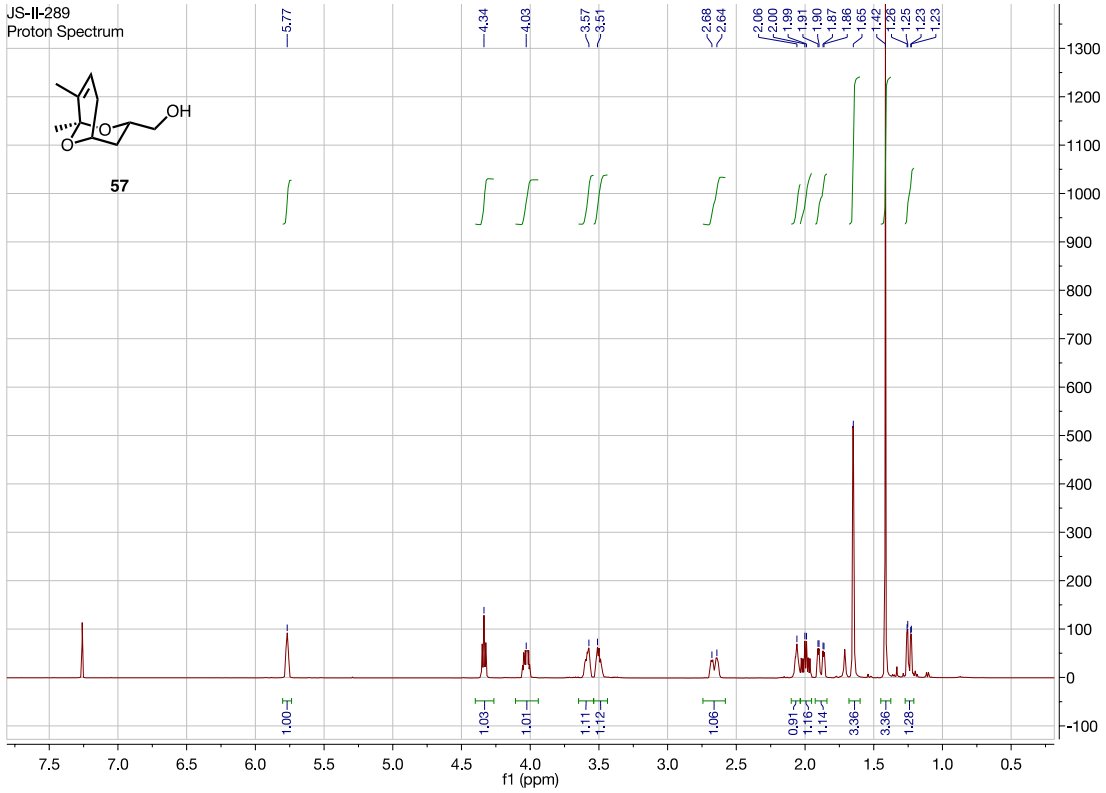
5.6 NMR Spectra

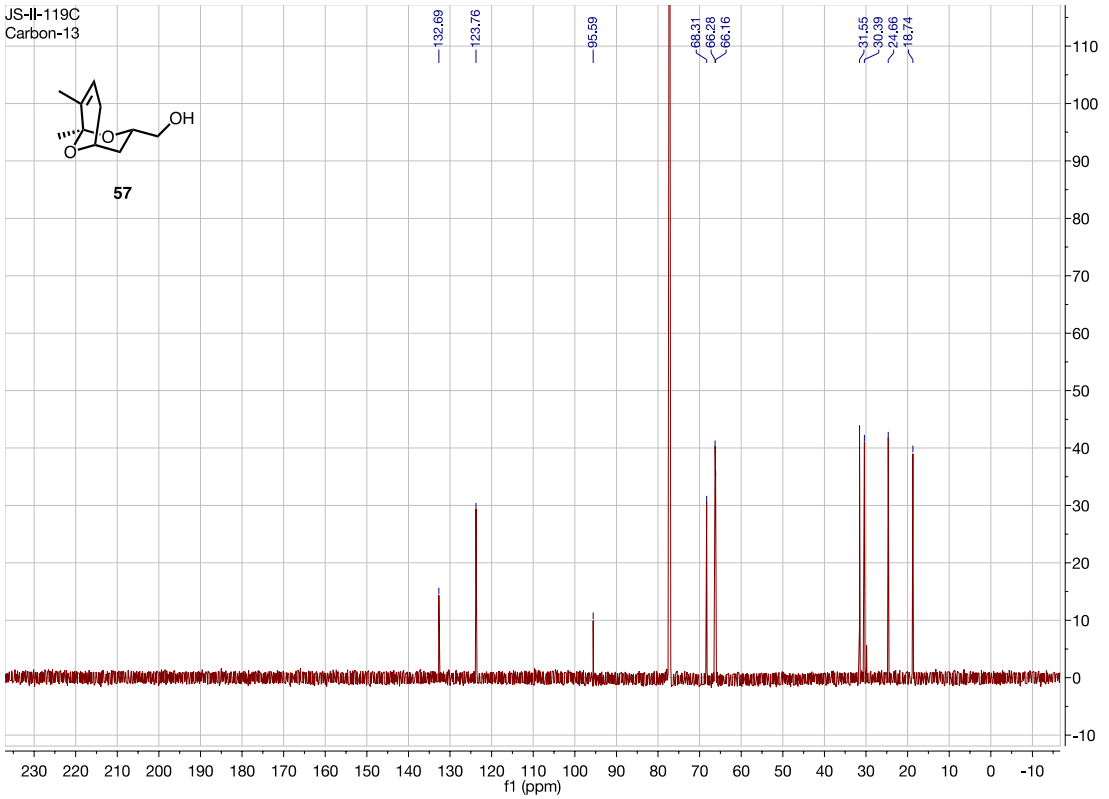


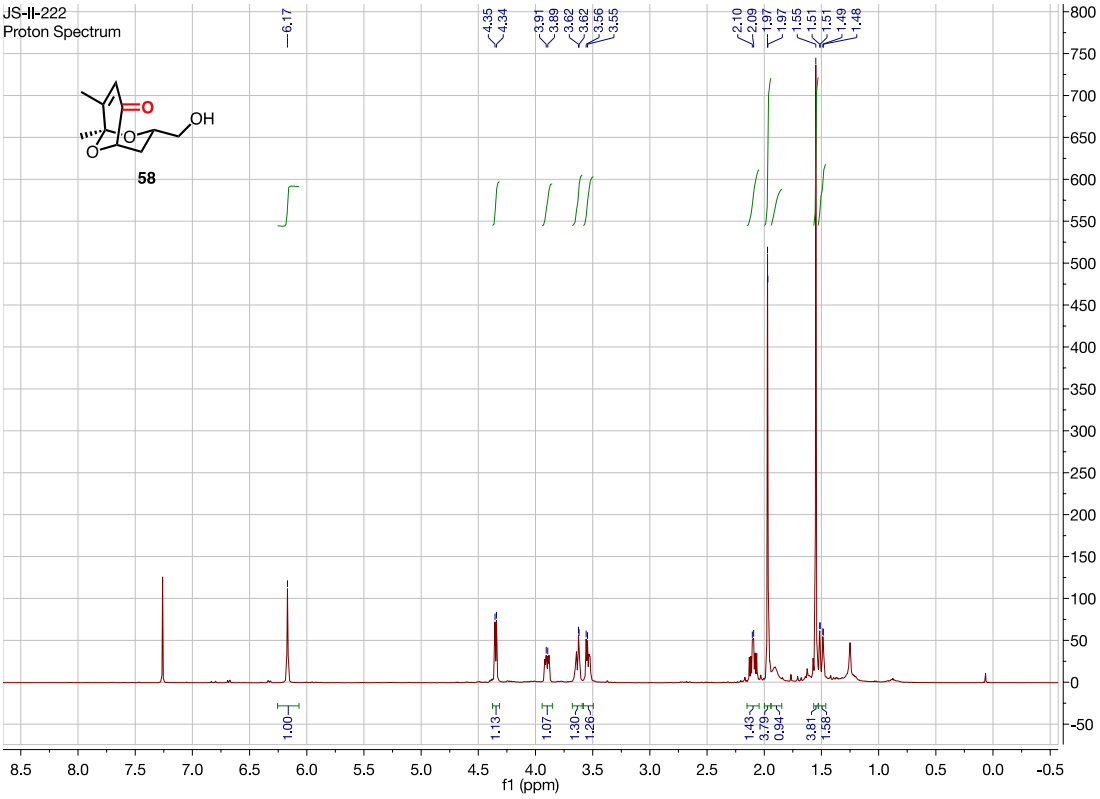


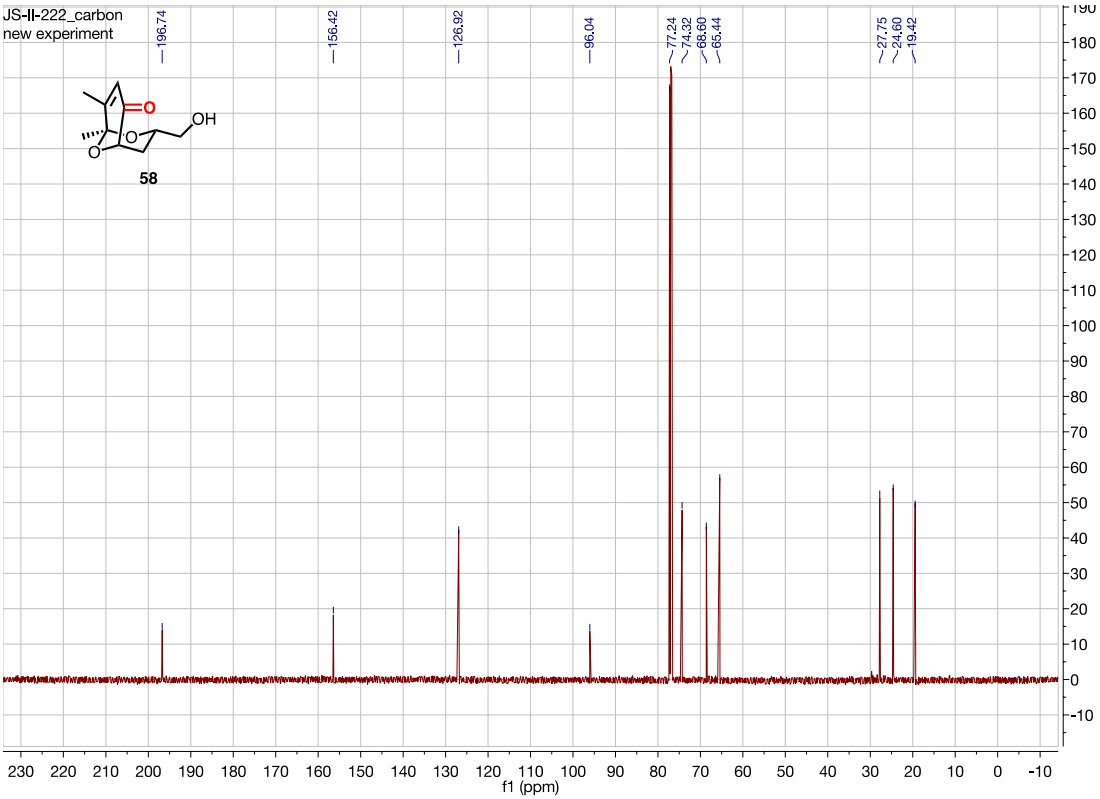

JS-II-261
Proton Spectrum
56
f1 (ppm)

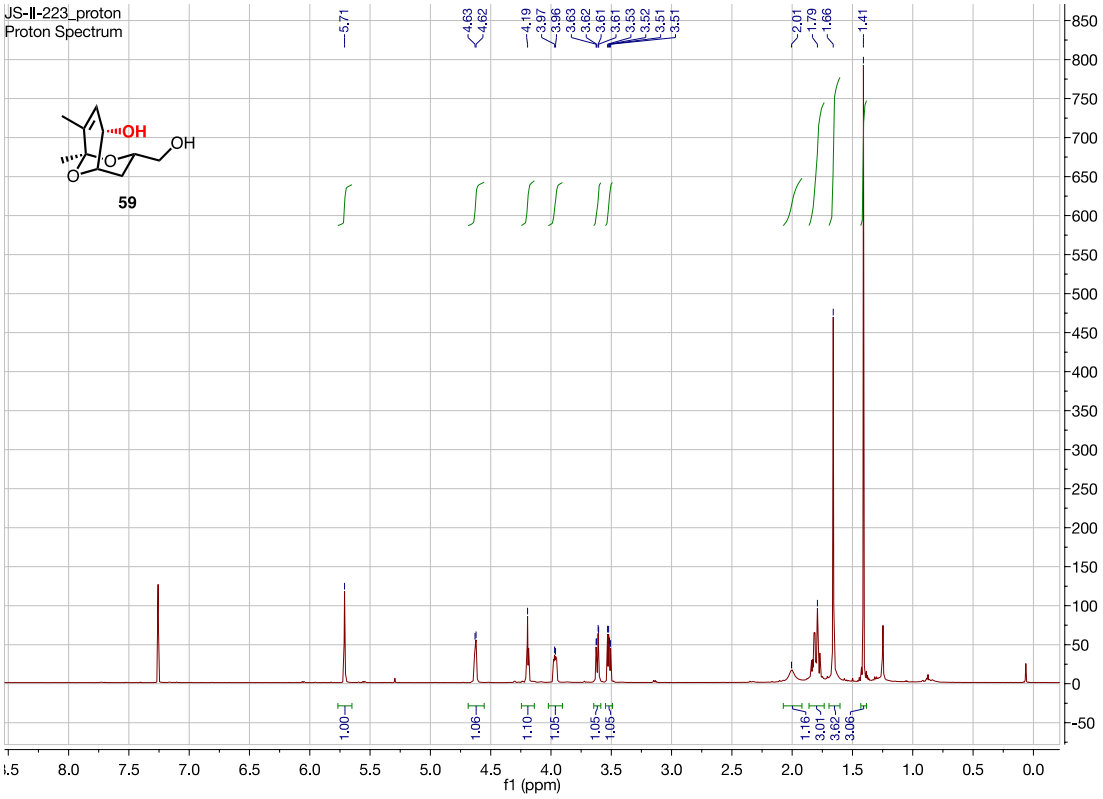

JS-II-261_carbon
Carbon-13
56
f1 (ppm)

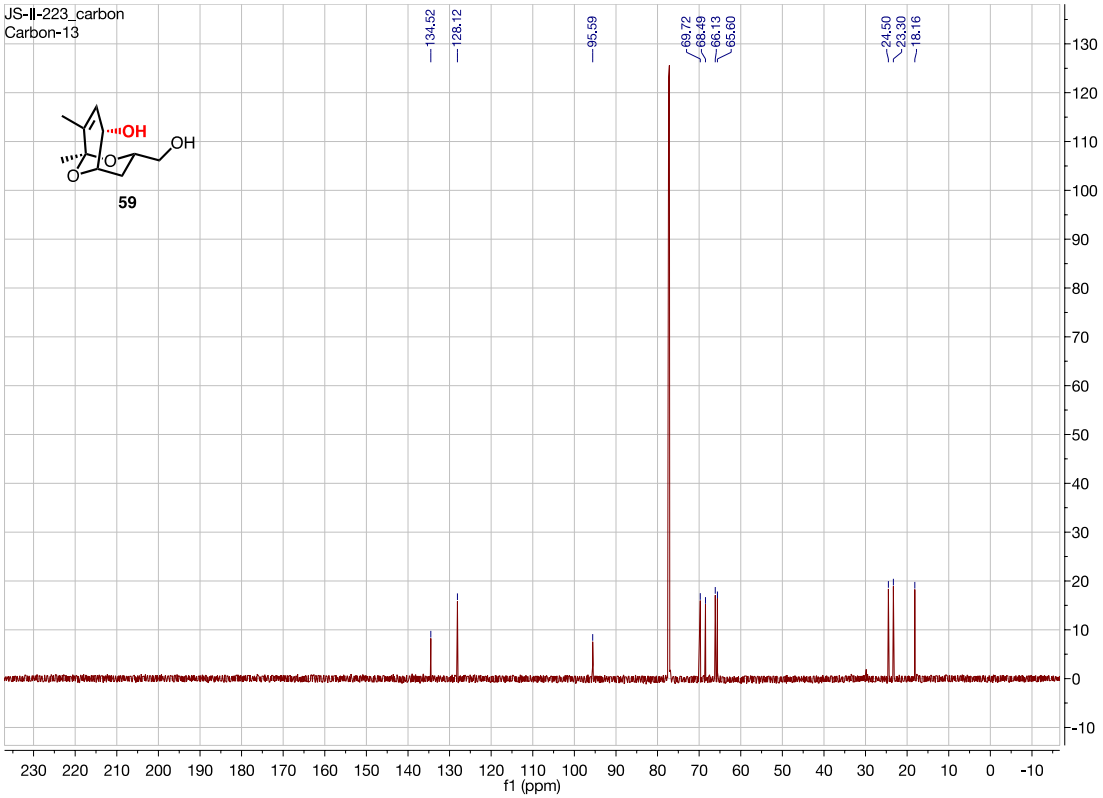

JS-II-289
Proton Spectrum
OH
O
O
57
5.77
4.34
4.03
3.57
3.51
2.68
2.64
2.06
2.00
1.99
1.91
1.90
1.87
1.86
1.65
1.42
1.26
1.25
1.23
1.23
1.00
1.03
1.01
1.11
1.12
1.06
0.91
1.16
1.14
3.36
3.36
1.28
f1 (ppm)

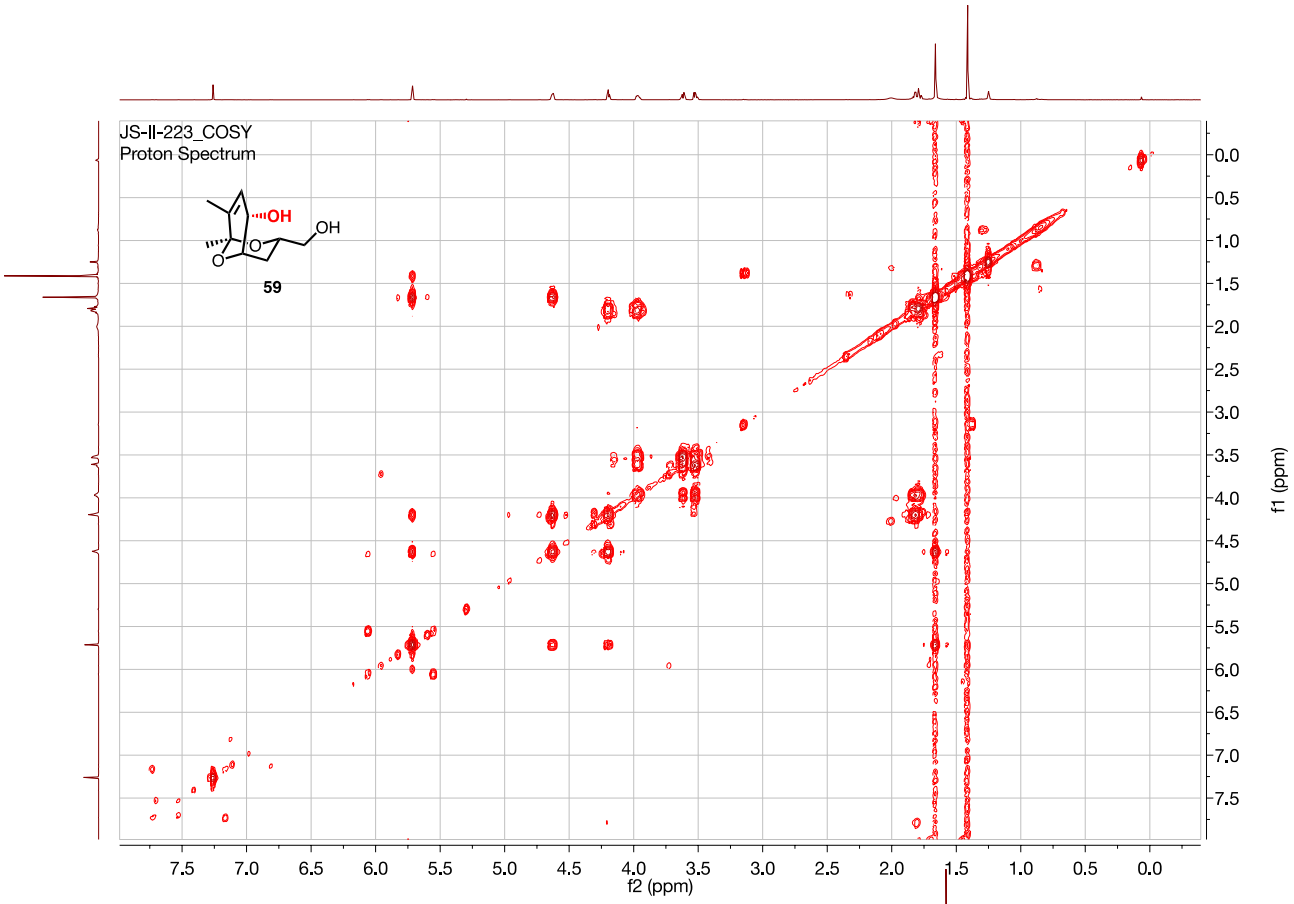

JS-II-119C
Carbon-13
OH
O
O
57
132.69
123.76
95.59
68.31
66.28
66.16
31.55
30.39
24.66
18.74
f1 (ppm)

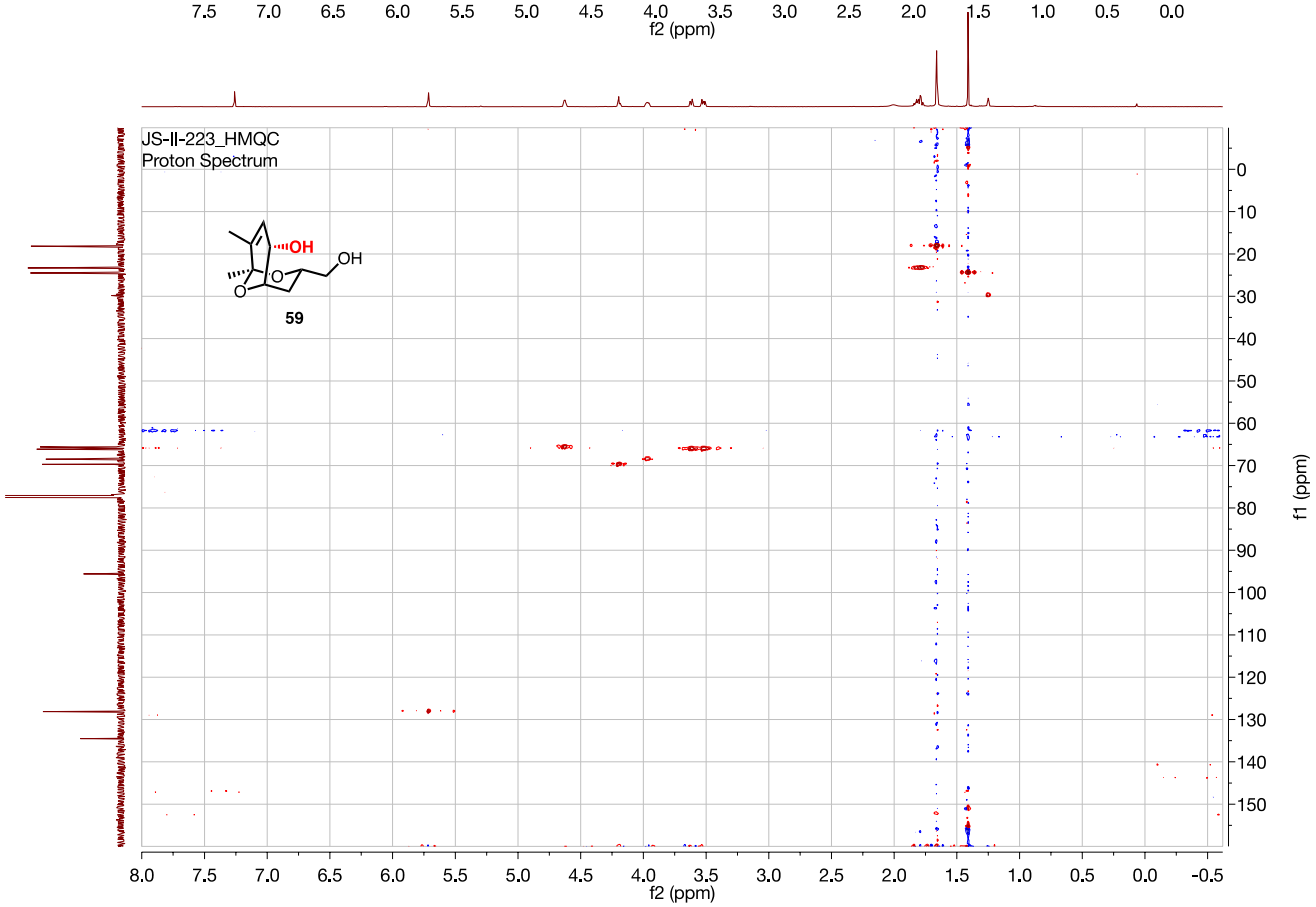

JS-II-222
Proton Spectrum
O
OH
O
O
58
6.17
4.35
4.34
3.91
3.89
3.62
3.62
3.56
3.55
2.10
2.09
1.97
1.97
1.55
1.51
1.51
1.49
1.48
1.00
1.13
1.07
1.30
1.26
1.43
3.79
0.94
3.81
1.58
f1 (ppm)

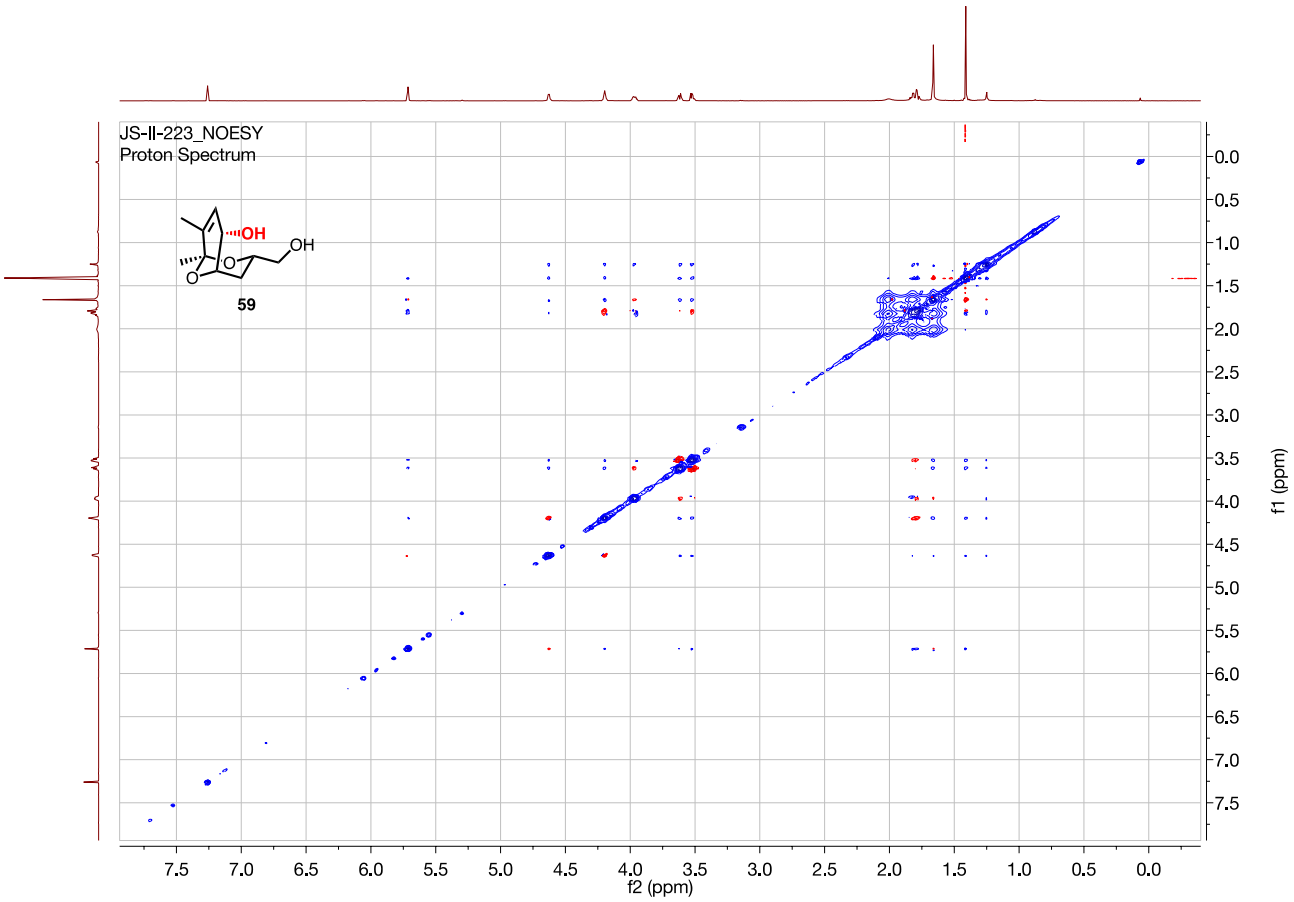

JS-II-222_carbon
new experiment
O
OH
O
O
58
196.74
156.42
126.92
96.04
77.24
74.32
68.60
65.44
27.75
24.60
19.42
f1 (ppm)

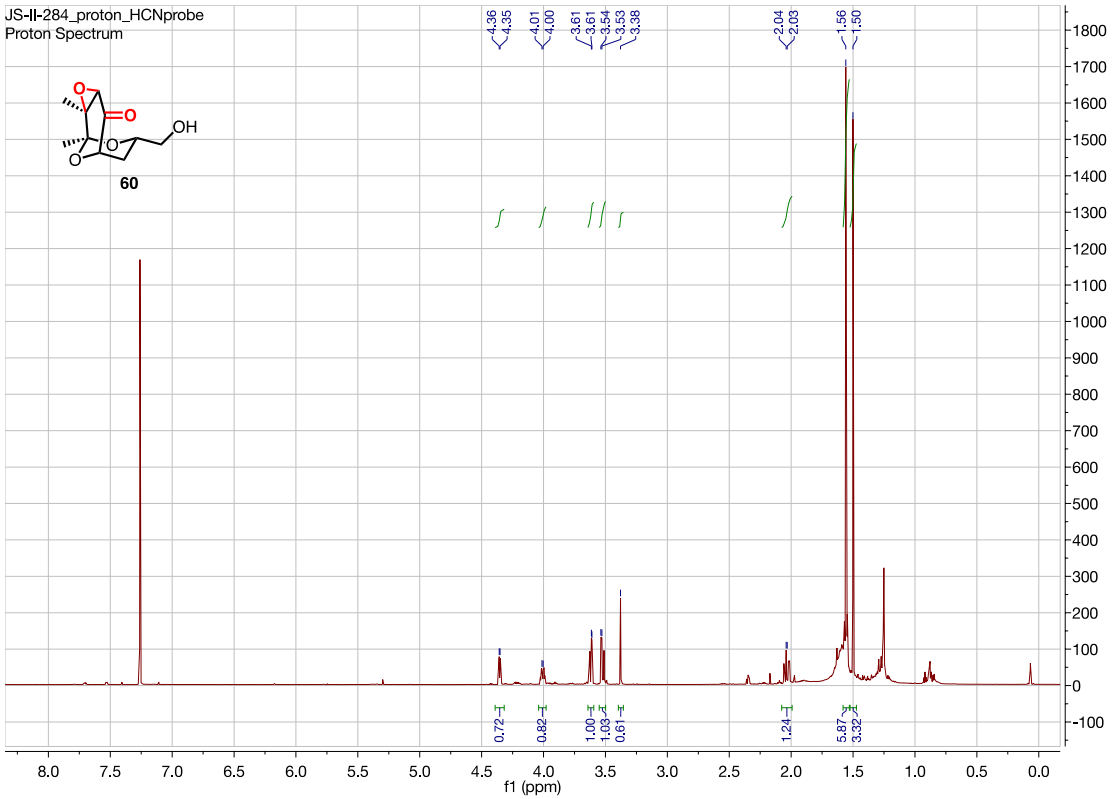

JS-II-223_proton
Proton Spectrum
OH
OH
O
59
5.71
4.63
4.62
4.19
3.97
3.86
3.83
3.82
3.81
3.53
3.52
3.51
3.51
2.01
1.79
1.66
1.41
1.00
1.06
1.10
1.05
1.05
1.05
1.16
3.01
3.62
3.06
f1 (ppm)

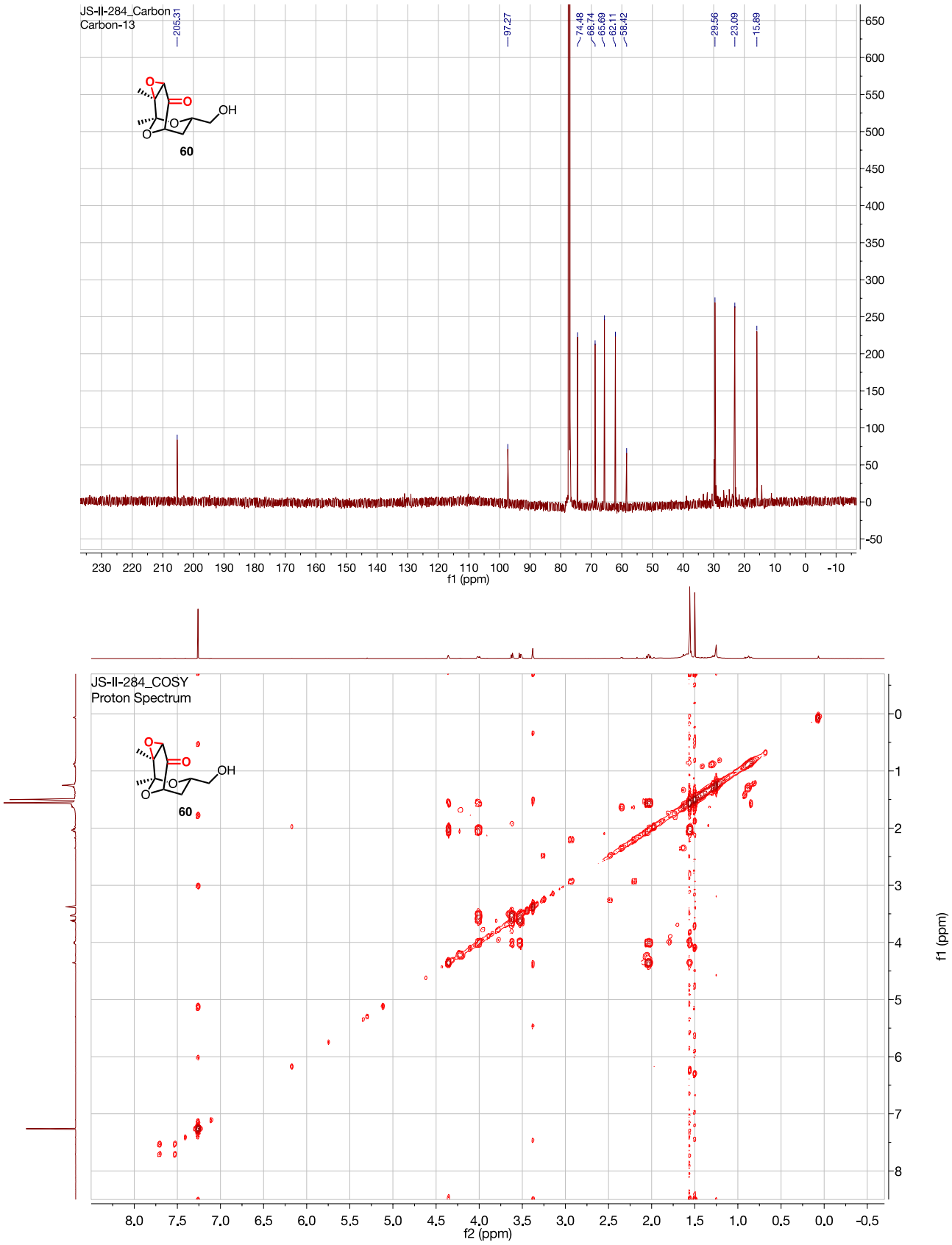

JS-II-223_carbon
Carbon-13
OH
OH
O
59
134.52
128.12
95.59
69.72
68.49
66.13
65.60
24.50
23.30
18.16
f1 (ppm)

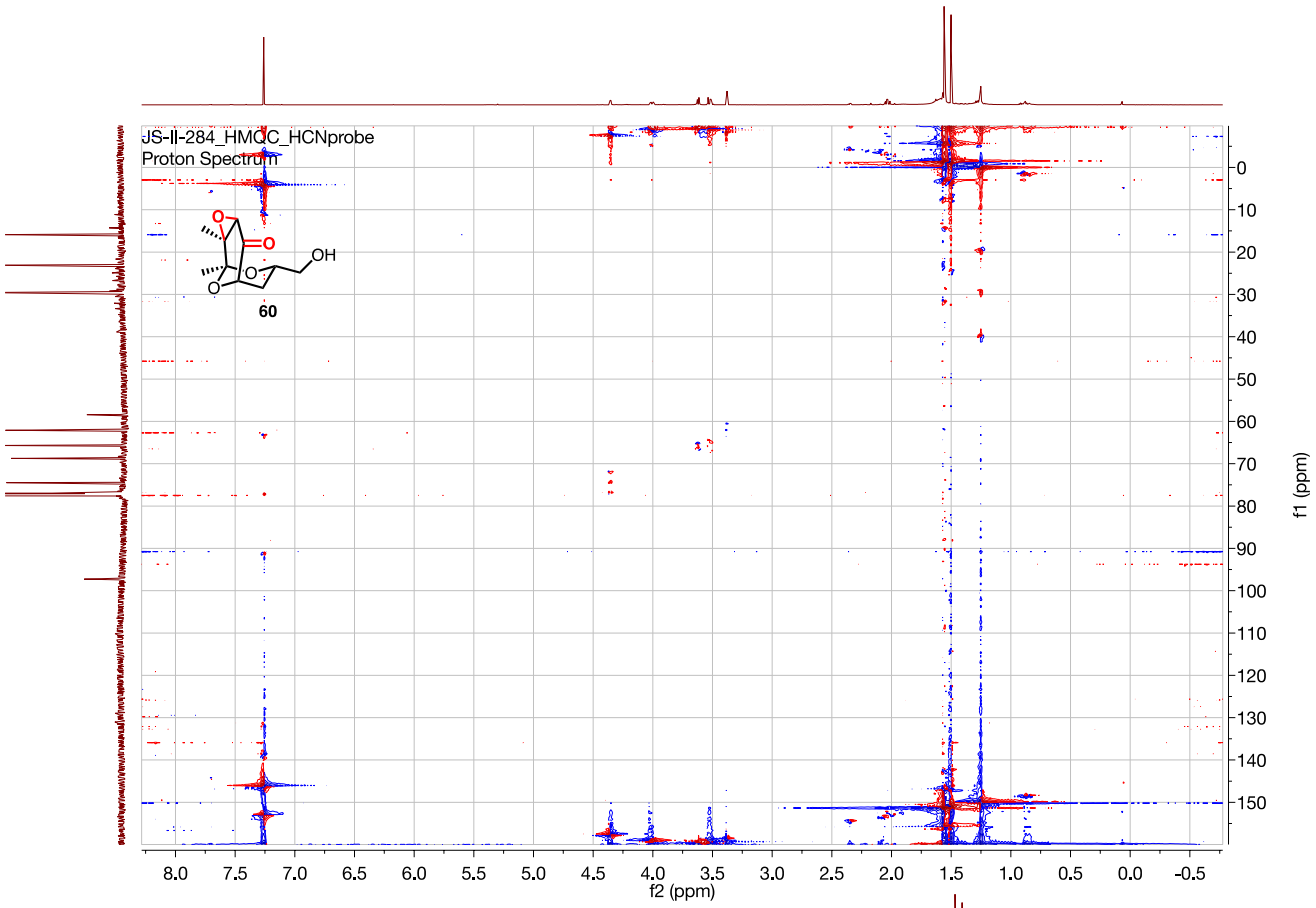

JS-II-223_COSY
Proton Spectrum
OH
OH
O
O
59
f2 (ppm)
f1 (ppm)

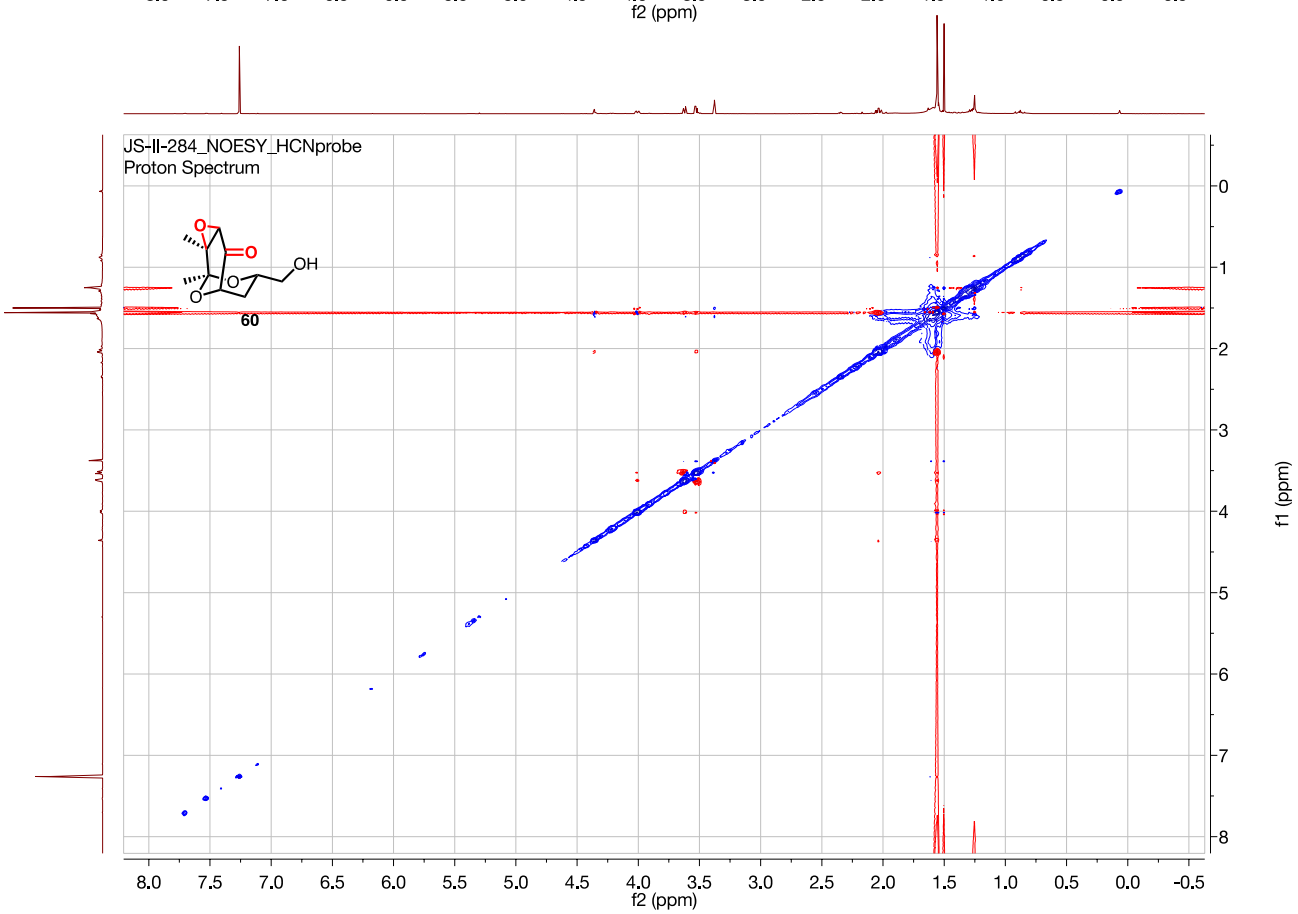

JS-II-223_HMQC
Proton Spectrum
OH
OH
O
O
59
f2 (ppm)
f1 (ppm)

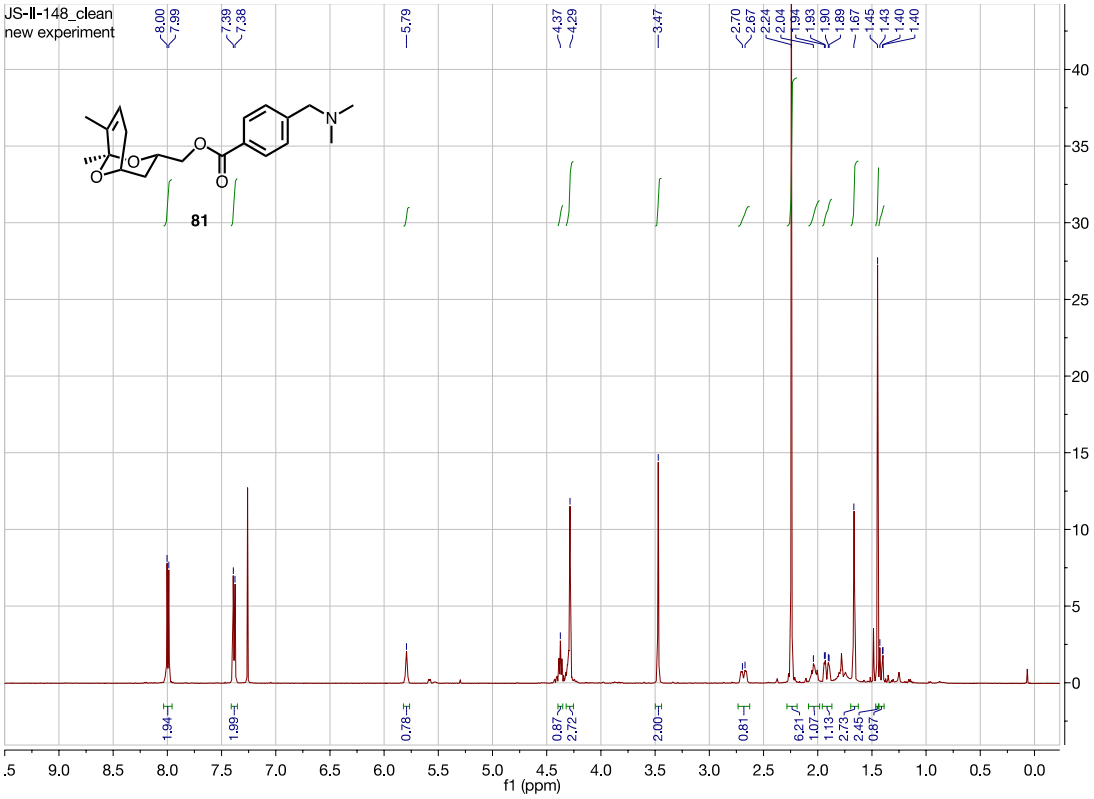

JS-II-223_NOESY
Proton Spectrum
OH
OH
59
f2 (ppm)
f1 (ppm)

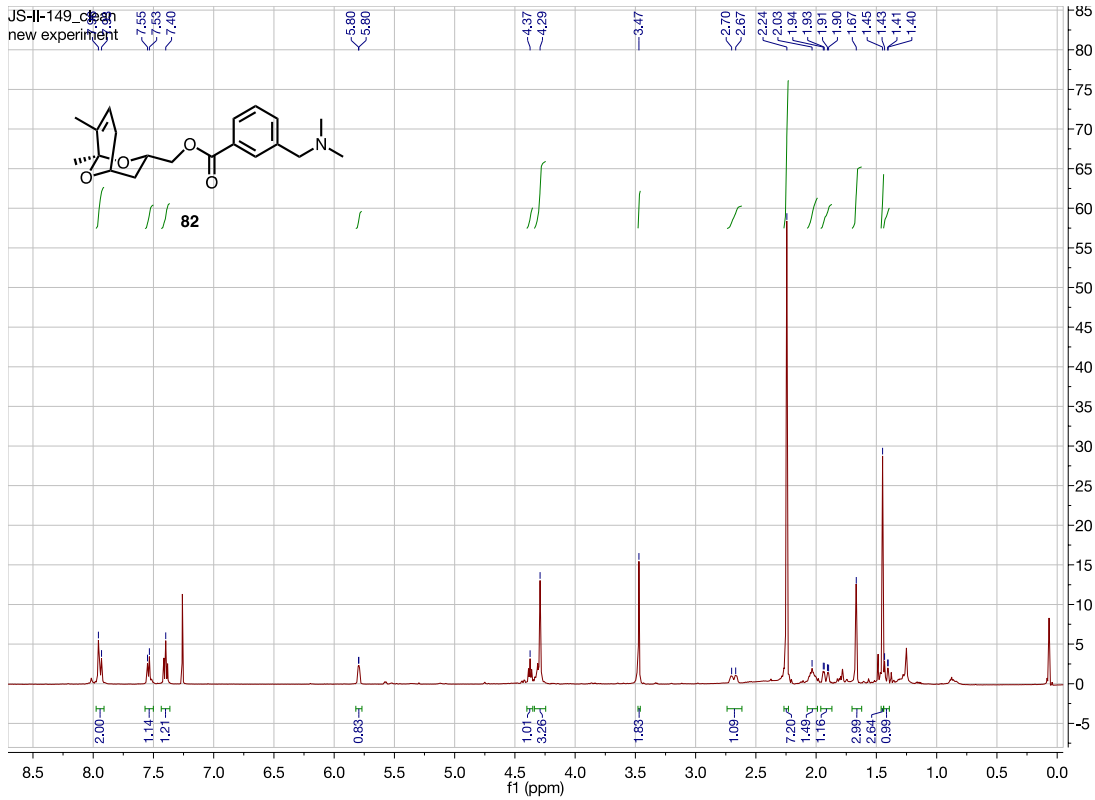

JS-II-284_proton_HCNprobe
Proton Spectrum
O
O
OH
60
4.36
4.35
4.01
4.00
3.61
3.61
3.54
3.53
3.38
2.04
2.03
1.56
1.50
0.72
0.82
1.00
1.03
0.61
1.24
5.87
3.32
f1 (ppm)

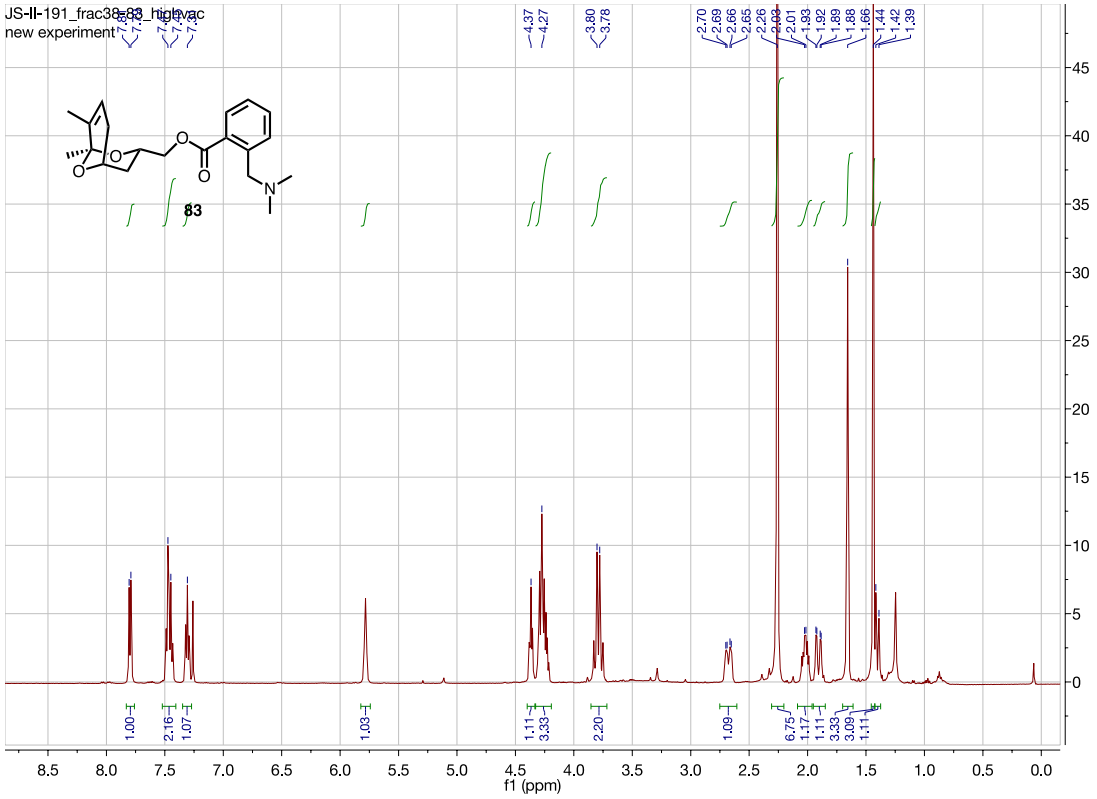

JS-II-284_Carbon
Carbon-13
205.31
97.27
74.48
68.74
65.69
62.11
58.42
29.56
23.09
15.89
OH
60
f1 (ppm)
JS-II-284_COSY
Proton Spectrum
OH
60
f2 (ppm)
f1 (ppm)

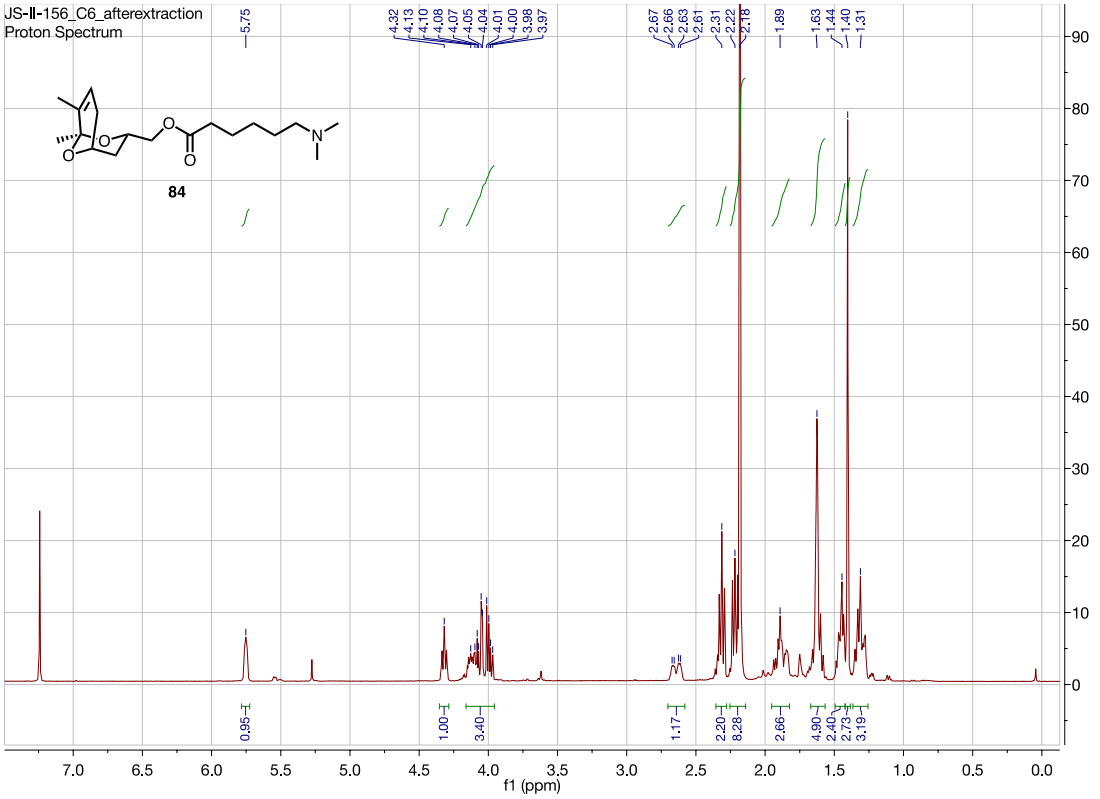

JS-II-284_HMQC_HCNprobe
Proton Spectrum
OH
60
f2 (ppm)
f1 (ppm)

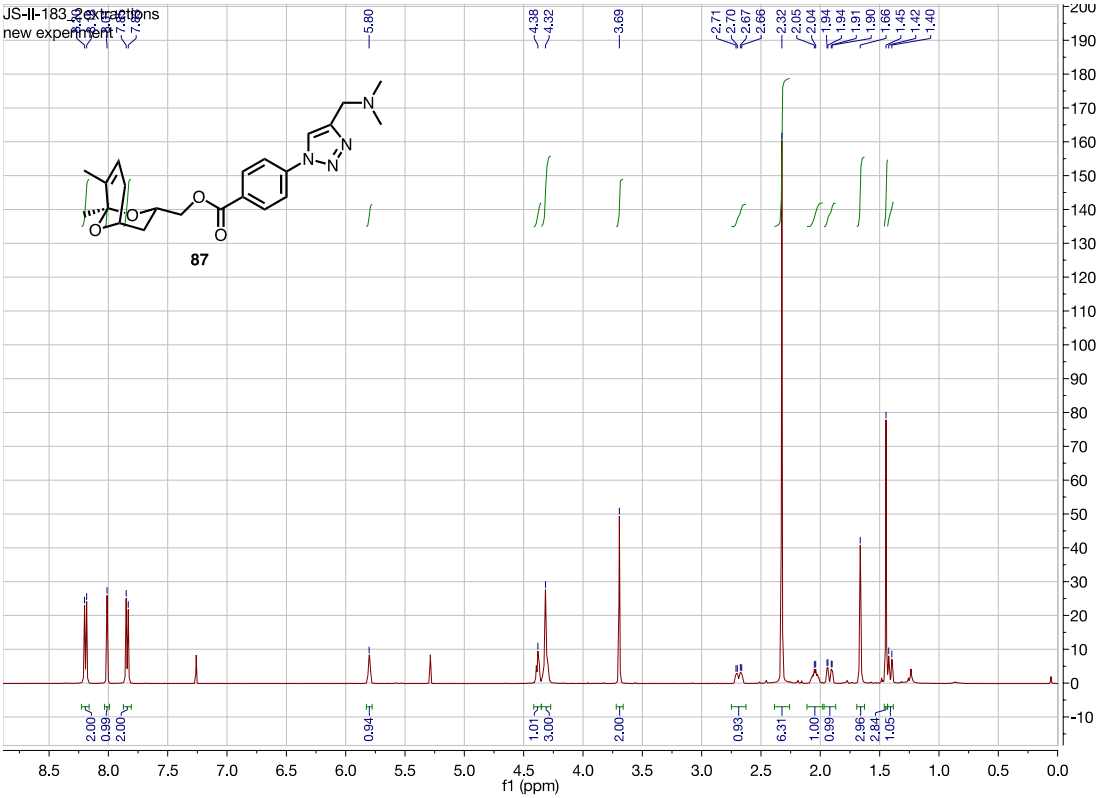

JS-II-284_NOESY_HCNprobe
Proton Spectrum
OH
60
f2 (ppm)
f1 (ppm)

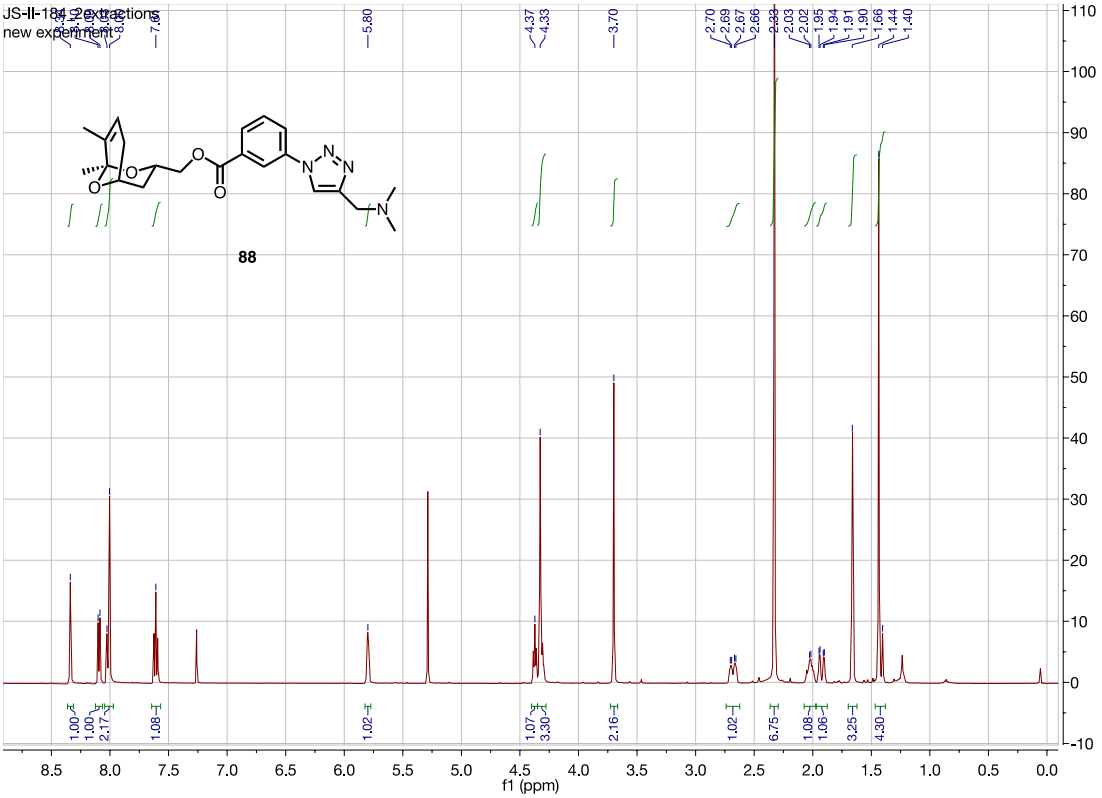

JS-II-148_clean
new experiment
81
f1 (ppm)

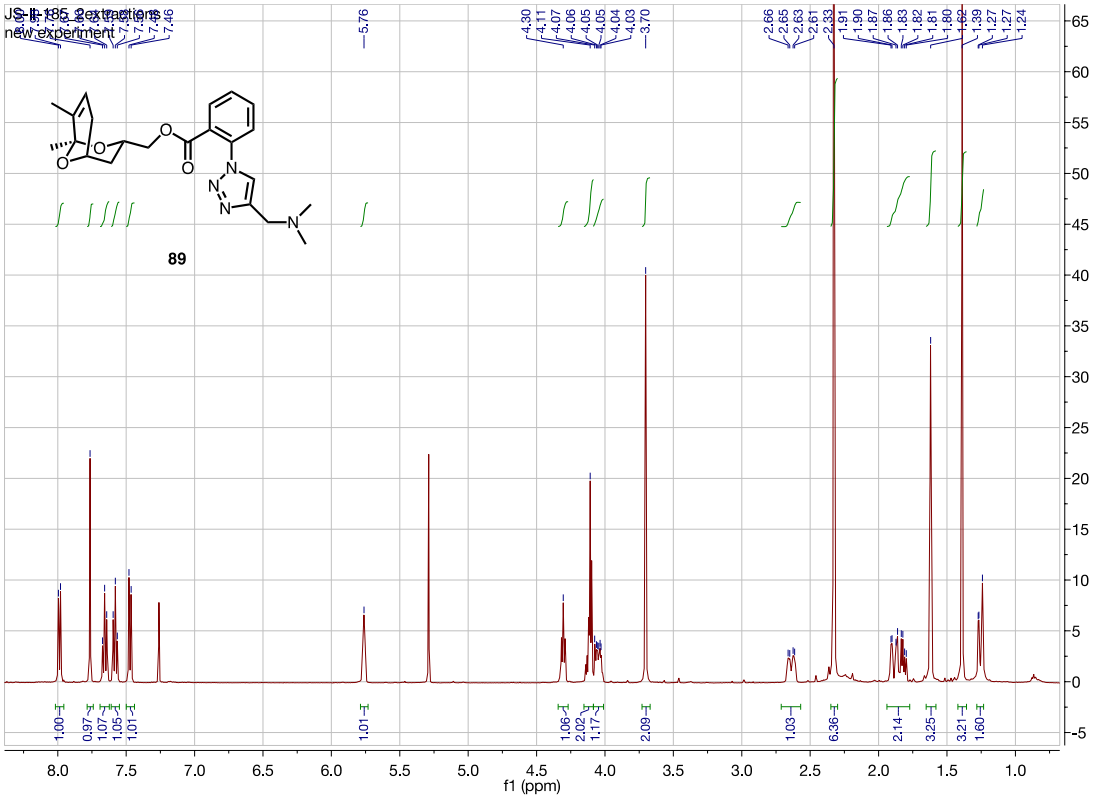

JS-II-149_clean
new experiment
82
f1 (ppm)

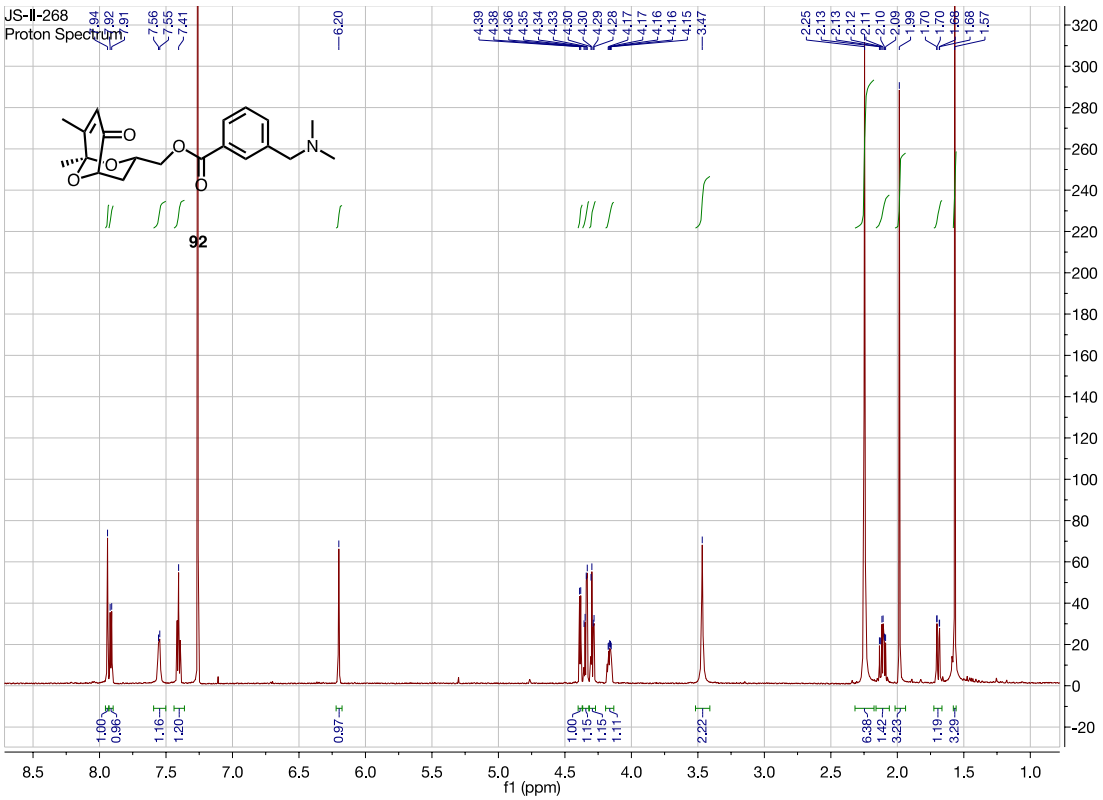

JS-II-191_frac38-83_highvac
new experiment
83
f1 (ppm)

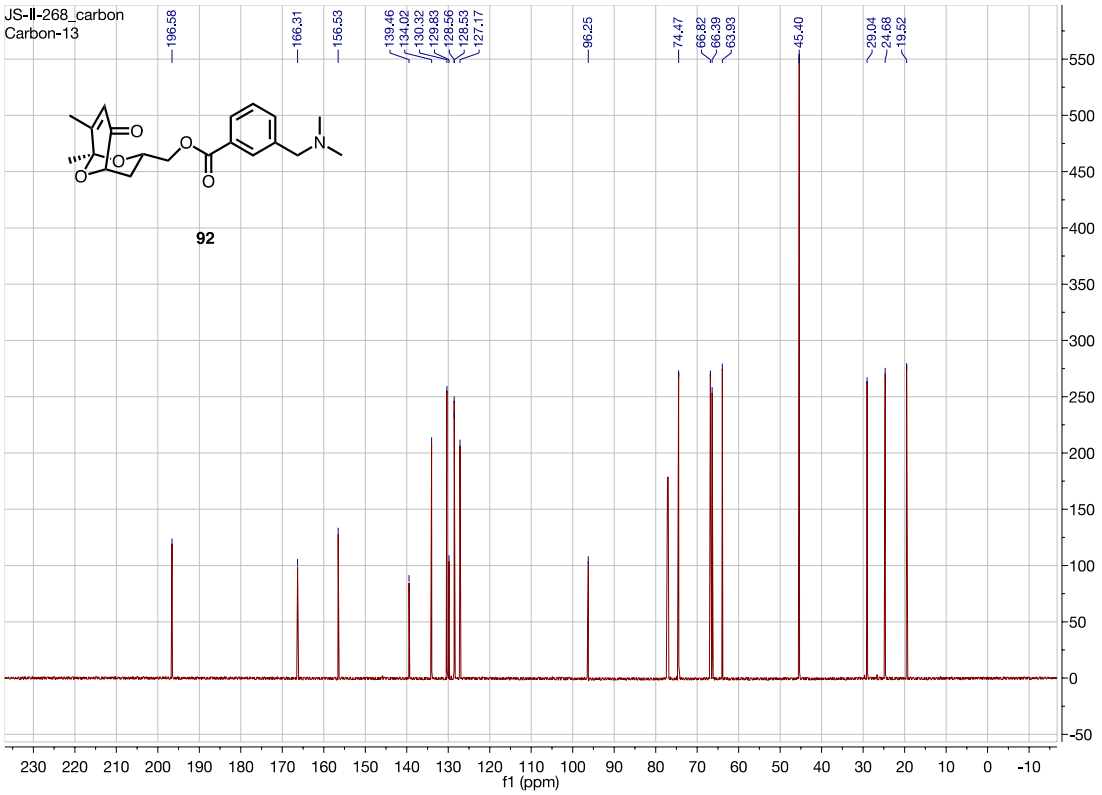

JS-II-156_C6_afterextraction
Proton Spectrum
84
f1 (ppm)

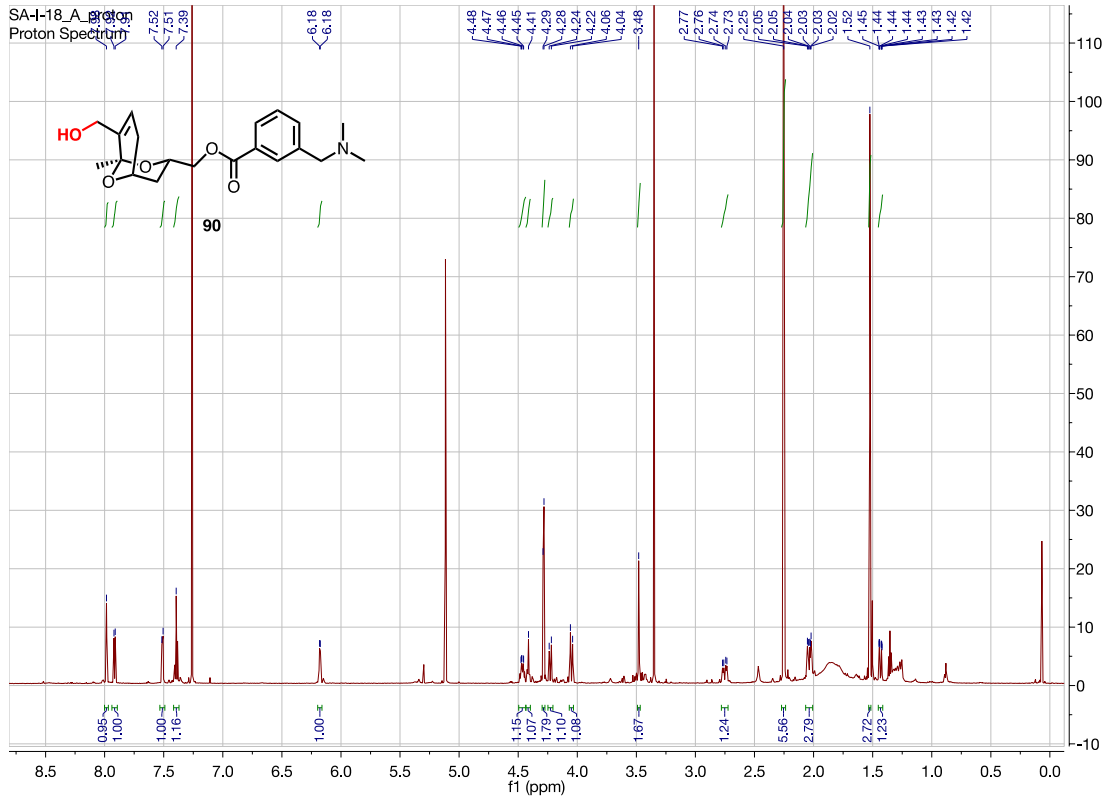

JS-II-183_2extractions
new experiment
87
f1 (ppm)

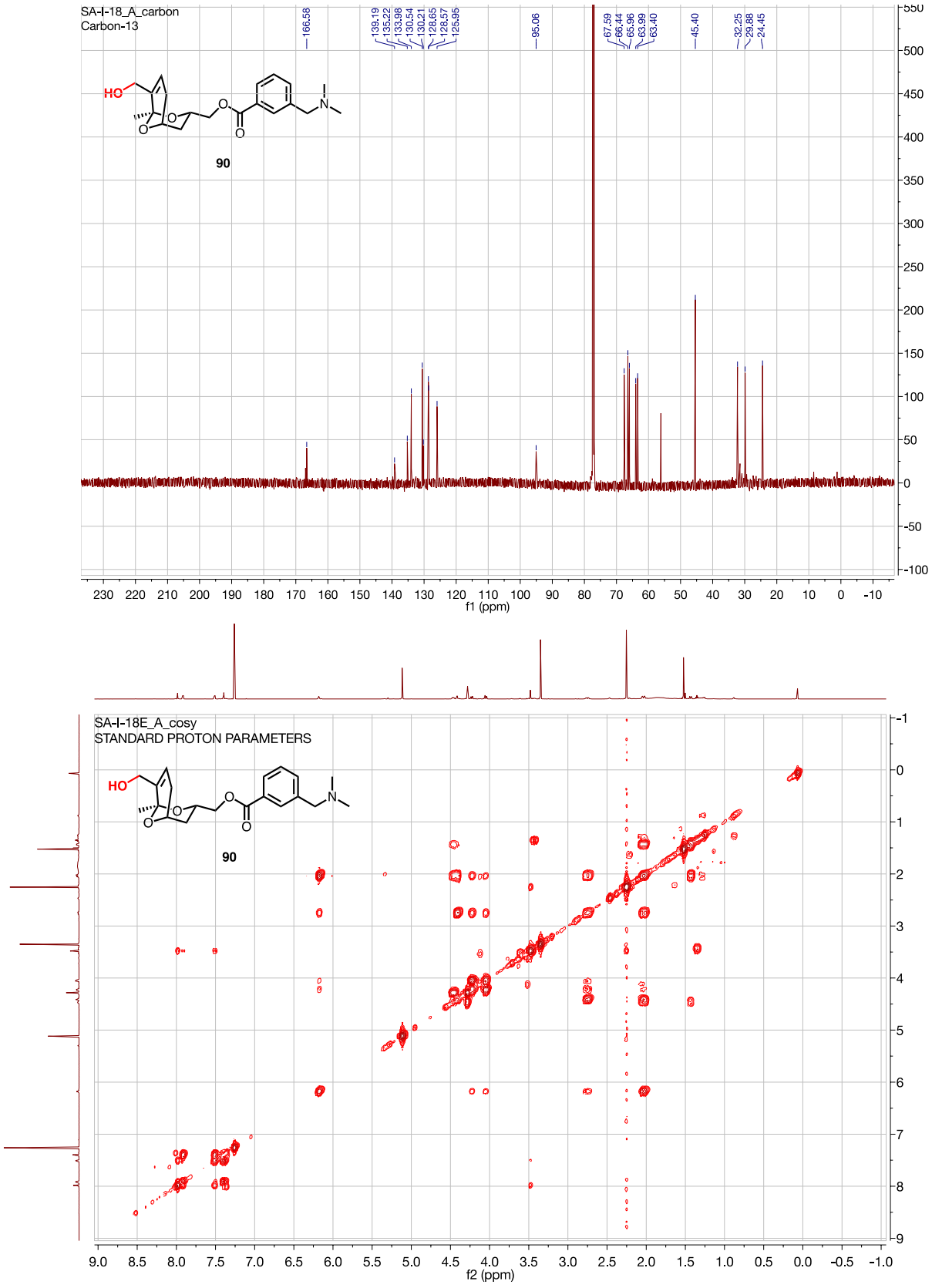

JS-II-184_2extractions
new experiment
88
f1 (ppm)

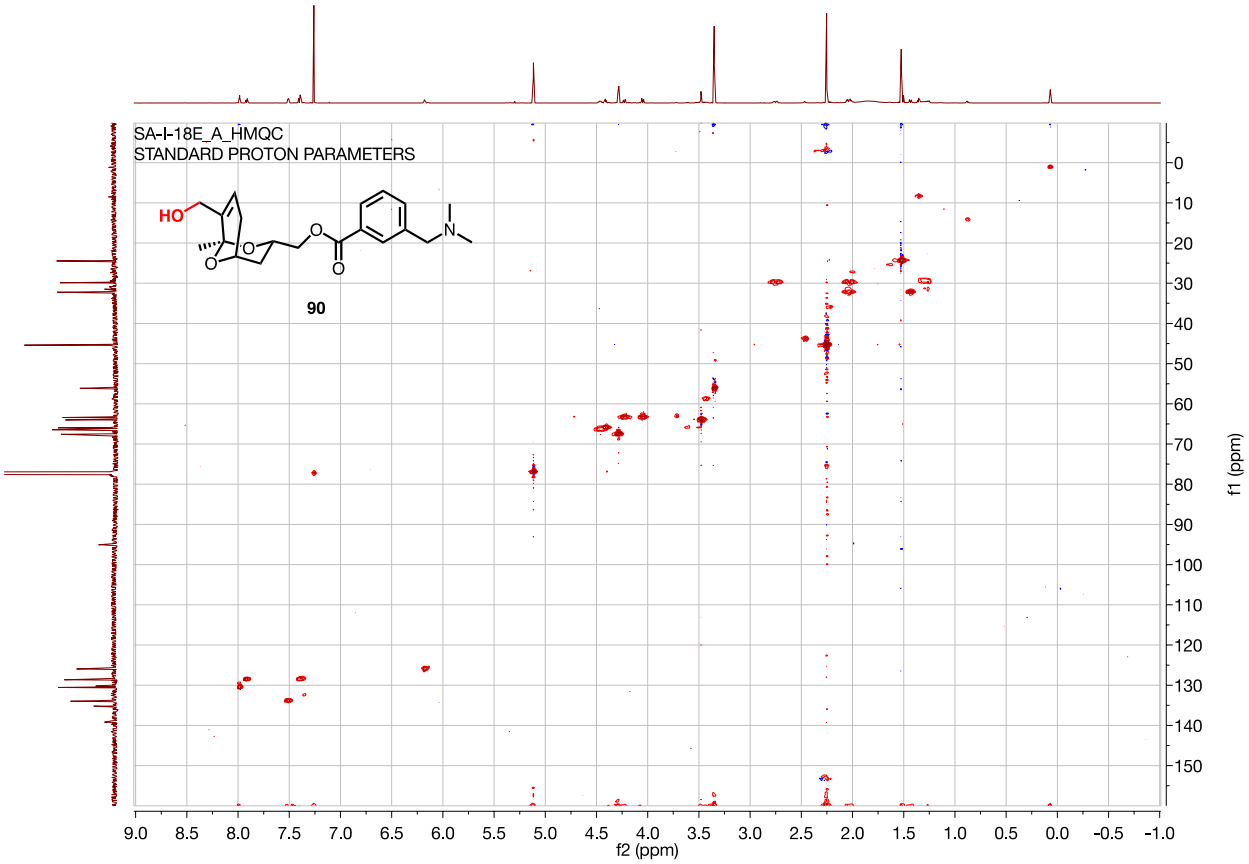

JS-II-185_2extractions
new experiment
89
f1 (ppm)

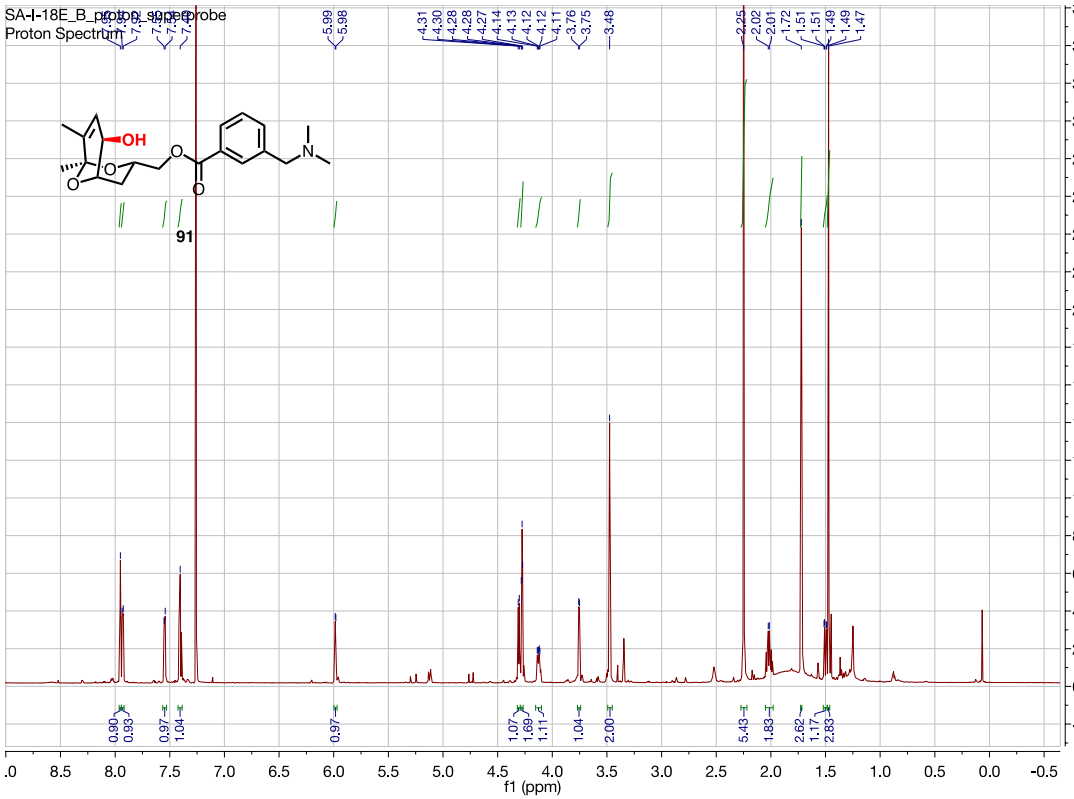

JS-II-268
Proton Spectrum
92
f1 (ppm)

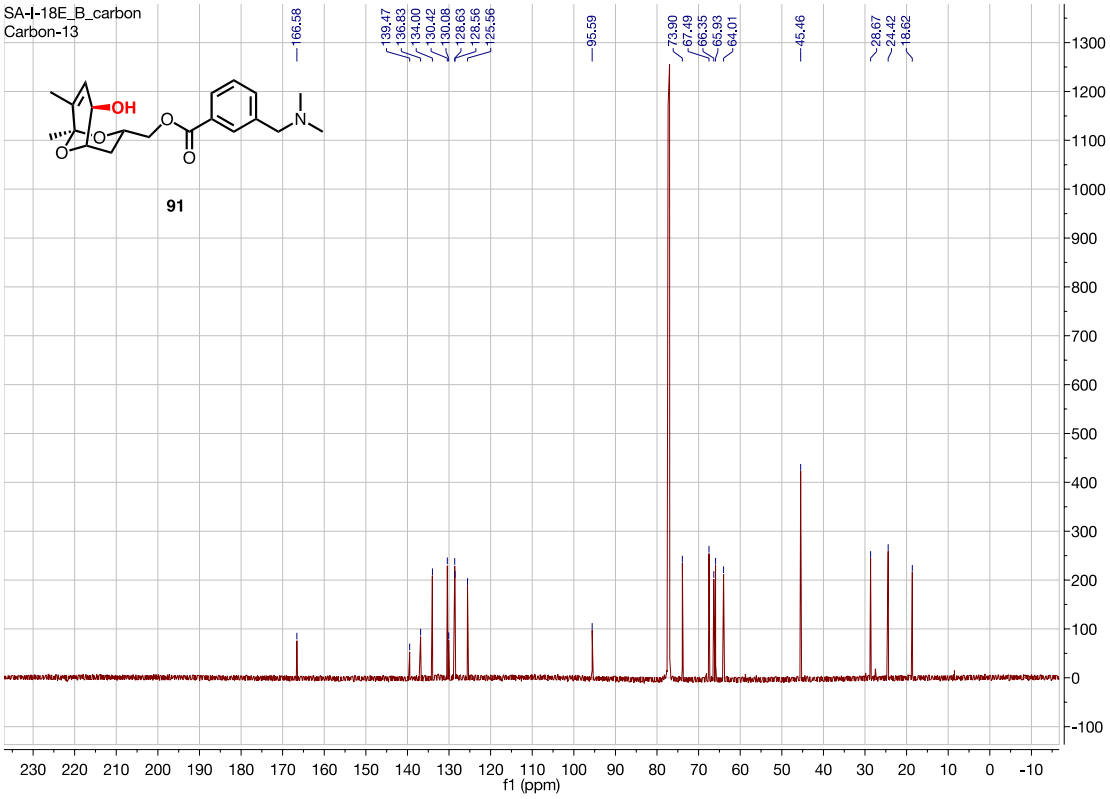

JS-II-268_carbon
Carbon-13
196.58
166.31
156.53
139.46
134.02
130.32
129.83
128.56
128.53
127.17
96.25
74.47
66.82
66.39
63.93
45.40
29.04
24.68
19.52
92
f1 (ppm)

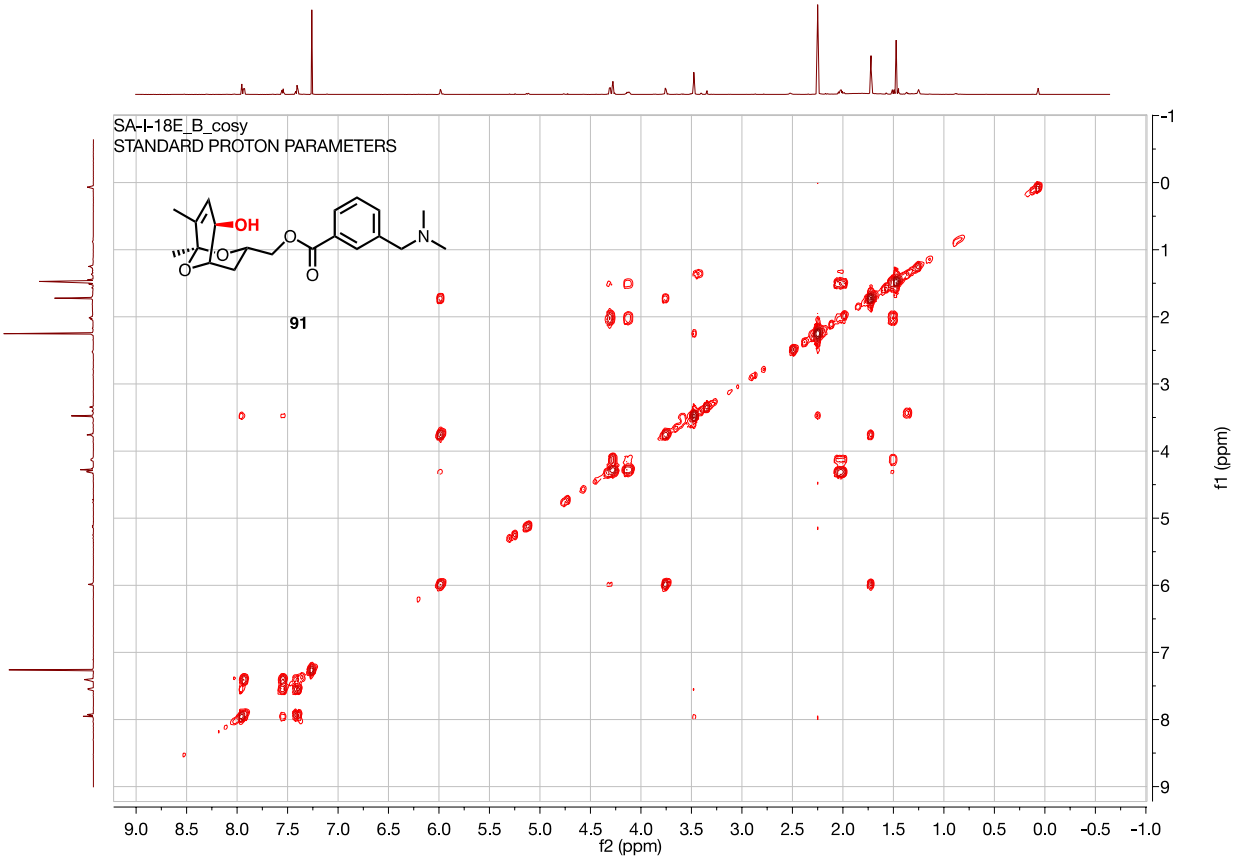

SA-I-18_A_proton
Proton Spectrum
7.52
7.51
7.39
6.18
6.18
4.48
4.47
4.46
4.45
4.41
4.29
4.28
4.24
4.22
4.06
4.04
3.48
2.77
2.76
2.74
2.73
2.25
2.05
2.05
2.04
2.03
2.03
2.02
1.52
1.45
1.44
1.44
1.44
1.43
1.43
1.42
1.42
HO
90
0.95
1.00
1.00
1.16
1.00
1.15
1.07
1.79
1.10
1.08
1.67
1.24
5.56
2.79
2.72
1.23
f1 (ppm)

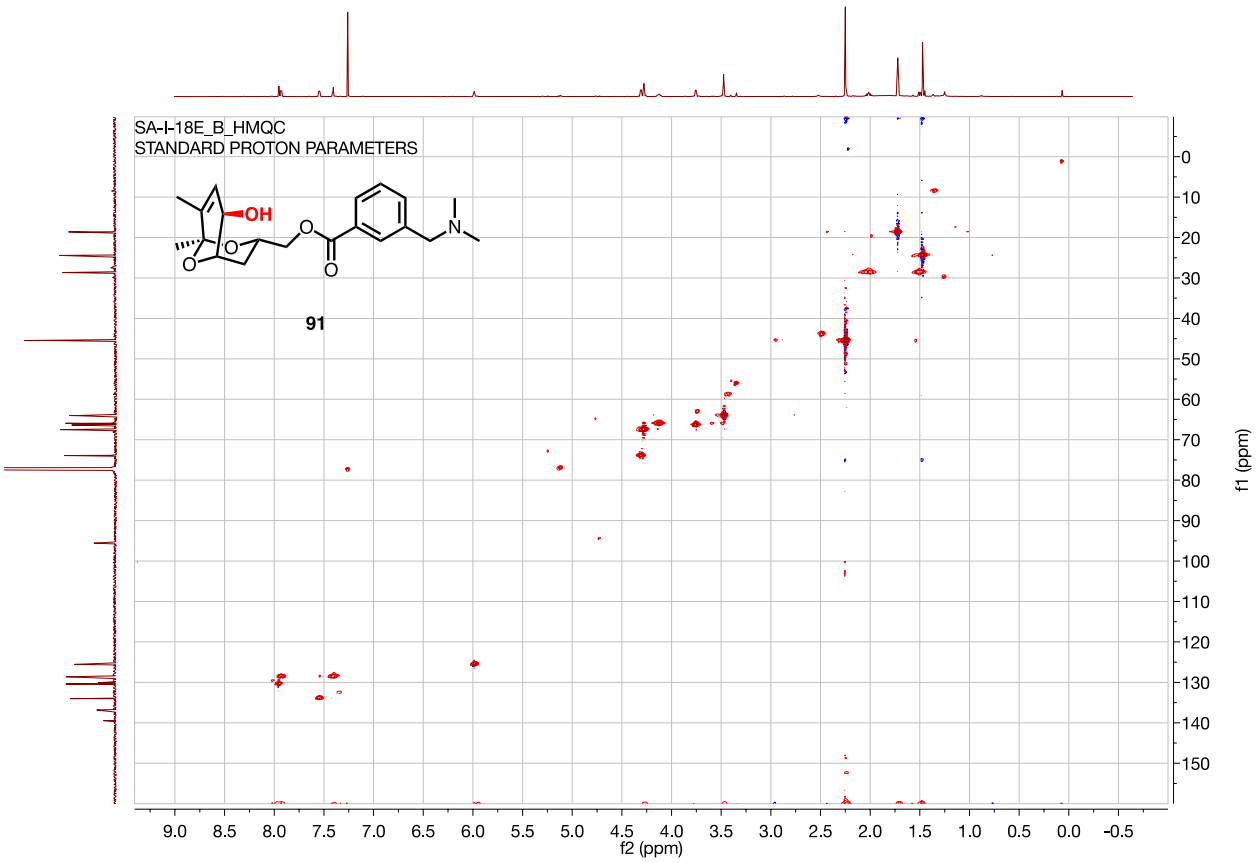

SA-I-18_A_carbon
Carbon-13
166.58
139.19
135.22
133.98
130.54
130.21
128.65
128.57
125.95
95.06
67.59
66.44
65.96
63.99
63.40
45.40
32.25
29.88
24.45
HO
O
O
O
O
N
90
f1 (ppm)
SA-I-18E_A_cosy
STANDARD PROTON PARAMETERS
HO
O
O
O
O
N
90
f2 (ppm)
f1 (ppm)

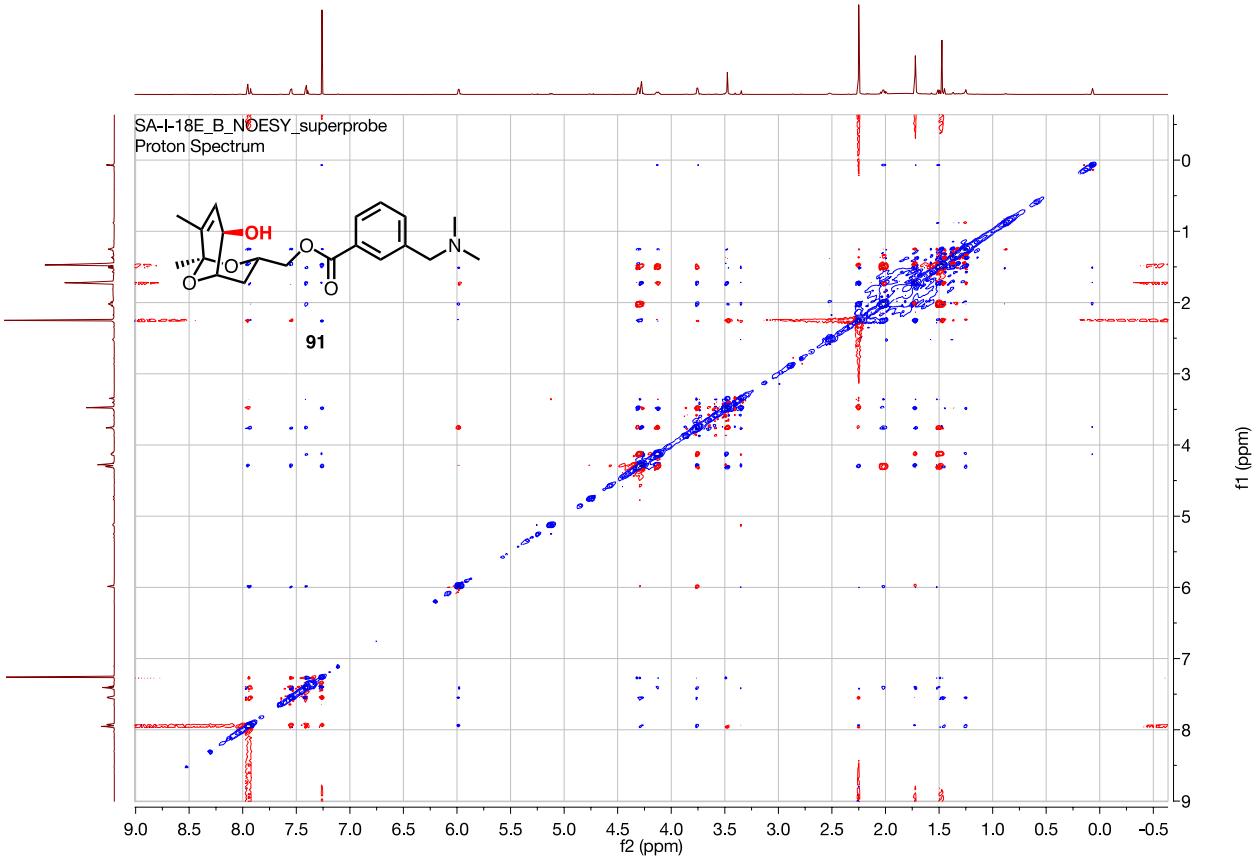

SA-I-18E_A_HMQC
STANDARD PROTON PARAMETERS
HO
O
O
O
O
N
90
f2 (ppm)
f1 (ppm)


SA-I-18E_B_proton_sgpeprobe
Proton Spectrum
OH
O
O
O
O
N
91
f1 (ppm)


SA-I-18E_B_carbon
Carbon-13
166.58
139.47
136.83
134.00
130.42
130.08
128.63
128.56
125.56
95.59
73.90
67.49
66.35
65.93
64.01
45.46
28.67
24.42
18.62
OH
O
O
O
N
O
91
f1 (ppm)


SA-I-18E_B_cosy
STANDARD PROTON PARAMETERS
OH
O
O
O
N
O
91
f2 (ppm)
f1 (ppm)


SA-I-18E_B_HMQC
STANDARD PROTON PARAMETERS
OH
O
O
O
O
N
91
f2 (ppm)
f1 (ppm)


SA-I-18E_B_NOESY_superprobe
Proton Spectrum
OH
O
O
O
O
N
91
f2 (ppm)
f1 (ppm)

5.7 Interview Questions

5.7.1 Hope College Visitation Interview Questions

Questions for PI (Dr. Johnson):

1. How did you decide that you want to be a professor at a primarily undergraduate institution (PUI) such as Hope College?
2. In your opinion, what are the primary differences between running a lab at a large, research-intensive university versus a small, primarily undergraduate college?
3. What are the biggest obstacles that you face as a PI at a small university and how do you do your best to overcome these challenges?
4. How do you overcome limitations in time, student experience and resources that are often stumbling blocks for many undergraduate research groups?
5. What is more important to you when running your lab: research or teaching?
6. Do you believe it is better to constantly supervise younger undergraduate researchers or to give them more independence to learn on their own?
7. How do you recruit and select new students to join your lab?
8. What are your criteria for selecting research projects?
9. What advice do you have for someone who hopes to someday be a PI of a lab like yours or who wants to modify the structure of an existing lab to be more “successful”?

Questions for Students:

1. Why did you decide to join Dr. Johnson's lab? What do you plan to do after you graduate?
2. Do you enjoy doing research? What do you like about it and what do you dislike?
3. Did you get to choose what project you're currently working on?
4. Do you agree with Dr. Johnson's mentoring style? What, if anything, would you change?
5. Do you enjoy working with the other people in your lab?
6. How do you feel you learn best, from your own mistakes or from advice from others? Do you prefer receiving advice from co-workers or from Dr. Johnson? Why?
7. Do you prefer working alone or collaboratively?
8. Do you feel you have enough independence in designing and engaging in your own original research? Do you ever want more guidance?
9. Do you feel comfortable working in the laboratory? Do you ever feel pressured or stressed? If so, why?
10. On a scale from 1-10, how important is publishing papers important to you?
11. What is the most important thing you've learned from joining Dr. Johnson's lab? What do you still want to learn?
12. How do you define success as a student researcher? What do you think has been the biggest key to your success as a research group, specifically with regards to your number of high impact publications?

13. What, if anything, would you change about the Johnson lab?

5.7.2 University of Michigan Visitation Interview Questions

Questions for Stanna (beginning of exchange program):

1. How did you become involved in the exchange program? What incentivized you to participate?
2. Do you enjoy doing research? What do you like about it and what do you dislike?
3. As of right now, what are your plans after you graduate from Hope College?
4. What are your expectations for working in the Montgomery lab? What do you hope to learn?
5. Do you have any concerns about working in a lab with graduate students or about the exchange program in general?
6. What is your opinion on the exchange program right now? Have you experienced any “stumbling blocks” or difficulties?

Questions for Stanna (halfway through exchange program):

1. How is working in the Montgomery lab going so far? Has it met your expectations?
2. What have you learned from working in the Montgomery lab? What do you still hope to learn?
3. Do you feel like you could have learned what you have learned so far by working in the Johnson lab again this summer? Do you miss anything about working in the Johnson lab?

4. What is your opinion of the Montgomery lab? What do you like about it and what do you dislike?
5. In your opinion, compare the similarities and differences between the Montgomery and Johnson labs.
6. Have you become a more or less confident researcher after joining the Montgomery lab?
7. Have you learned anything from your interactions with lab members that is not directly related to laboratory skills or content knowledge?
8. What is your opinion on the exchange program right now? Have you experienced any “stumbling blocks” or difficulties?

Questions for Stanna (end of exchange program):

1. Describe your overall experience working in the Montgomery lab this summer.
2. Did the exchange program meet your expectations? Are you glad you participated?
3. What did you learn from working in the Montgomery lab? If there anything that you wished you had learned that you didn’t?
4. Do you feel like you could have learned what you learned by working in the Johnson lab again this summer? Did you miss anything about working in the Johnson lab?
5. What is your opinion of the Montgomery lab? What did you like about it and what did you dislike?
6. In your opinion, compare the similarities and differences between the Montgomery and Johnson labs.

7. Have you become a more or less confident researcher after working in the Montgomery lab?
8. Did you learn anything from your interactions with lab members that is not directly related to laboratory skills or content knowledge?
9. Do you think your feelings about research have changed after working in the Montgomery lab? Have your career plans changed at all?
10. What is your overall opinion on the exchange program? Did you experience any "stumbling blocks" or difficulties?
11. If you could change anything about the exchange program what would you change?

Questions for Stanna (~2 months after completion of exchange program):

1. Have you felt that the exchange program has helped you in your time since you've been back to Hope? If so how?
2. Have any of your thoughts/feelings about the program changed since we last spoke?

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